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Capillary Electrophoresis Fractionation, Enzyme Chemistry, and Mass Spectrometry for Sensitive Detection and Identification of Peptides and Proteins

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Capillary Electrophoresis Fractionation, Enzyme Chemistry, and Mass Spectrometry for Sensitive Detection and Identification of Peptides and Proteins

Running Title: CE-MALDI-MS for Protein Identification

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Background

Detection and, particularly, identification of agents of biological origin (ABO) (i.e., protein toxins, bacteria, and viruses) require sophisticated analytical techniques. Considering the chemical composition of these agents, it is clear that all ABOs contain peptides and proteins. Because of significant differences in genomes that govern the expression of peptides and proteins in different organisms, the identities of peptides and proteins are expected to be very different for different ABOs. Thus peptides and proteins can be used as biomarkers for ABO detection. An analytical technique capable of detection peptides and proteins with high sensitivity and specificity can potentially be used as a single platform for the detection of all ABOs.

In the past decade or so, mass spectrometry has been rapidly advanced and it has now become the method of choice for peptide and protein analysis in many applications. Recently several research groups have proposed and illustrated the possibility of using MS for bacteria differentiation. The potential advantages of MS for ABO detection and identification include high sensitivity, high specificity and relatively high speed. Another significant advantage of MS over other techniques is the ability of MS to identify unknown proteins, hence the possibility of using MS for the detection of unknown ABOs. In this regard, MS should be particularly valuable for identification of unknown protein toxins where recent advancement of genetic engineering has made possible to create and produce diverse and potentially very harmful peptide and protein toxins.

For MS to become a truly robust and routinely useful platform for ABO detection, there are several areas of improvements that we still need to work on. One major issue is related to detection sensitivity. The amount of toxins or the number of bacteria cells or virus particles collected from a field can be very small. MS must be able to detect them without laborious and time-consuming cell growth or other special sample handling processes.

In the past several years, my laboratory has been focused on the development of MS front-end devices for the improvement of MS detection sensitivity. We have demonstrated the state-of-the-art detection for peptides and proteins. With microspot matrix-assisted laser desorption ionization (MALDI), we can detect peptides with a total sample loading of 25,000 molecules (42 zeptomoles) and proteins with a total sample loading of several attomoles. We have also demonstrated the possibility of carrying out protein analysis and protein identification from a single cell with a cell diameter of less than 7 μm. However, all these analyses were done with simple peptide and protein mixtures where one component is the predominant species (e.g., in red blood cell, hemoglobin is the predominant protein).

For ABO analysis, we are dealing with complex mixtures. We need to use a separation method to isolate the proteins of interest and then do protein identification by MS. Among several separation methods, only capillary electrophoresis (CE) and related capillary separation techniques such as capillary chromatography and capillary electrochromatography can handle trace amounts of samples with high separation efficiency. Thus, we proposed to develop a

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method based on CE for fractionation of proteins, followed by peptide mapping with MS.

The long-term goal of this research is to develop an advanced mass spectrometric method combined with separation and enzyme chemistry for ABO detection and identification. The specific goals of the research in the past two years (April 1999 to March 2001) for this particular project were:

(1) To develop an integrated method based on protein fractionation by capillary electrophoresis, followed by off-line enzyme digestion, and then mass spectrometry analysis of the enzyme digests and protein database searching for protein identification;

(2) To demonstrate and apply this method for the identification of proteins from bacterial samples and other samples provided by DRES;

(3) To compare this method with other mass spectrometric approaches such as on-line LC or CE-ESI-MS.

We have completed the proposed work and met the research goals. Two manuscripts based on this work have been submitted for publications. Another manuscript is in preparation. We gave a conference presentation each year at the American Society for Mass Spectrometry annual conference (2000 and 2001) on this work.

In the following three sections, more detailed descriptions of our work are presented. Part 1 describes our initial effort in demonstrating the possibility of combining CE fractionation with the nanoliter chemistry station for enzyme digestion, followed by MALD-MS analysis of the digest for peptide mass mapping for protein identification. This preliminary study also suggested that there was a need for further optimization of the method for efficient protein separation as well as developing more robust interface to MALDI for multiple component fractionations. Part 2 presents a study of using a surfactant-treated capillary for efficient CE separation of proteins and demonstrates the advantages of this type of separation for CE fractionation and subsequent MALDI analysis. Comparison of our method with other techniques is also discussed. Part 3 describes our journey in the course of developing and applying CE/MALDI-MS for bacterial protein analysis for a cell extract of *E. coli*. This work represents, to our knowledge, the first demonstration of CE/MALDI-MS for complex protein sample analysis. It shows that CE/MALDI-MS is a promising technique for proteomics applications. However, our work also reveals several issues that need to be addressed. Some planed future work is discussed.

I would like to thank Dr. Paul D'Agostino of the Defence Research Establishment at Suffield for helpful discussion during the course of this work.

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Part 1: Protein Identification at a Sub-picomole Level Using Microliter Volume Capillary Electrophoresis Fractionation Combined with MALDI-MS Peptide Mass Mapping

1.1. Summary

A microliter volume capillary zone electrophoresis (CZE) fraction collection method is described. The steps in the optimization of this method are presented along with practical considerations for its combination with matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis. Nanoliter chemistry techniques for protein digestion combined with mass assignment of whole proteins by direct MALDI-MS analysis allows complete protein characterization from a single CE fraction containing less than 1 pmol protein.

1.2. Introduction

Capillary electrophoresis has emerged as an important tool in protein analysis. It is by nature a small-scale technique, and the low sample load required for a run makes it attractive for the analysis of biological samples or other situations where limited sample is available. The sensitivity of matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) makes it compatible with the analyte levels used in CE, and the two techniques have been combined in various configurations. In some cases the capillary effluent is deposited by different mean directly onto the MALDI target [1-4], and in other cases it is collected into capillaries or vials [5-8]. Most commercial CE instruments are capable of collecting fractions in the latter manner.

Off-line coupling of CE and MALDI-MS has the advantage of allowing multiple analyses on the collected fractions. As a result, the analysis routine can be tailored to suit the sample. For example, different enzymatic digestions or derivatization reactions can be used to help characterize different proteins. With off-line CE, however, sample dilution during fraction collection can make further analysis of subpicomole amounts of protein difficult. We have developed a microliter volume fraction collection method that keeps sample dilution to a minimum and allows fractions from a single CE run to be used for both peptide mapping and direct MALDI-MS analysis. The method is straightforward and can easily be used with commercial CE instruments.

1.3. Experimental

1.3.1. Reagents.

Standard proteins, trypsin and α-cyano-4-hydroxycinnamic acid (HCCA) were purchased from Sigma-Aldrich (St. Louis, MO). The HCCA was purified by recrystallization from ethanol before use. The CE buffer was purchased from Agilent Technologies (Palo Alto, CA). The water used to prepare all solutions was deionized using a Milli-Q water purification system (Millipore, Worchester, MA).

1.3.2. CZE.

Figure 1.1 shows a schematic of the CE fractionation setup. CZE was performed on an Agilent ^{3D}CE instrument with ChemStation software for instrument control and data acquisition. Polyvinyl alcohol (PVA) coated capillaries (100 μM ID, 33.4 cm total length; Agilent Technologies) were used for all separations. The capillary was conditioned before each run by flushing with 10 mM phosphoric acid for 1 min followed by running buffer (50 mM sodium phosphate, pH 2.5) for 2 min. A 40 nL portion of aqueous protein sample was injected at 50 mbar for 1 s. A buffer plug was injected for 2 s after the sample to avoid sample loss due to thermal expansion once voltage was applied. Separation was carried out at 20°C with a running voltage of 155 V/cm.

Peaks were detected with the instrument's diode array detector and automatically collected using the built-in fraction collection macros. A stainless steel cap was made to extend the outlet electrode for electrokinetic fraction collection. No modification was necessary for hydrodynamic fraction collection. Fractions were collected either at running voltage or with 50-mbar pressures. If not used immediately, the fractions were transferred to siliconized polypropylene microvials and frozen.

1.3.3. MALDI.

MALDI analysis of whole proteins was performed on an HP G2024A TOF mass spectrometer, and analysis of tryptic digests was performed on a homebuilt linear time-lag focusing time-of-flight mass spectrometer that has been previously described [9,10]. Typically, 100-200 laser shots were summed for each mass spectrum.

A three-layer deposition technique was used for CE fractions containing whole proteins. The target is prepared by drying a thin first layer of matrix (0.13 M HCCA in 33% methanol/acetone) on the target followed by a thicker second layer (saturated HCCA in 30% methanol/water). The sample is then deposited on the matrix, allowed to dry, and rinsed with a drop of water that is removed under a stream of air. The tryptic digest solutions were deposited as described below.

1.3.4. Tryptic Digestion.

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Digests for peptide mapping were carried out using a nanoliter chemistry station that has been previously described [10]. Briefly, the nano ChemStation consists of a 20 μM ID silanized fused-silica capillary connected to a disposable syringe and mounted on a XYZ manipulator with video microscope observation. A volume of CE fraction is drawn into the capillary, and the solvent is evaporated with the aid of a nitrogen stream across the capillary tip. This step is repeated until the desired amount of protein is concentrated inside the capillary. 10-20 nL of each CE fraction was typically required for peptide mapping. Buffered trypsin solution is then drawn into the capillary, the end is sealed against a Parafilm-covered holder, and the mixture is allowed to digest for 20 min. For MALDI analysis, 1 nL of second layer matrix solution is drawn into the capillary and deposited along with the digestion mixture onto a matrix-covered target.

1.4. Results and Discussion

The two main modes of mobilization for fraction collection with CE are electrokinetic (using voltage) and hydrodynamic (using pressure). Electrokinetic mobilization has the advantage of maintaining the characteristic plug-like flow profile of CE so that the separation resolution is not decreased during fraction collection. To maintain the voltage, an electrolyte solution must be used in the collection vial. With the regular instrument configuration, a minimum of 17 μL of solution was required to provide contact with the electrode. A stainless steel cap was made to extend the electrode to within approximately 1 mm of the end of the capillary. With this configuration, the collection volume was lowered to 2 μL.

The use of dilute hydrochloric acid has been reported as an effective fraction collection solution for peptides with less MALDI signal suppression than phosphate buffer solutions [11]. The 5-50 mM solutions of hydrochloric, phosphoric and trifluoroacetic acids evaluated in this experiment, however, caused fluctuations in the CE current and absorbance readings that interfered with fraction collection. Running buffer (50 mM sodium phosphate) was found to the most effective as the collection solution. Rinsing with water on the MALDI target or between drying steps with the nanoliter chemistry station removed most of the buffer salts from the fractions.

Using electrokinetic fraction collection, proteins could be characterized from as little as 5 pmol injected on the CE instrument. With smaller amounts of protein, however, increased pH at the outlet electrode due to the electrolysis of water significantly decreased protein recovery and made mass spectrometric analysis irreproducible. Lower detection limits were achieved with hydrodynamic fraction collection. Figure 1.2 shows that molecular weight could be determined from 200 fmol of protein injected on CE. Peptide mapping could be performed with as little as 300 fmol of protein (Figure 1.3). Since an electrolyte is not required for pressure collection, 1.5 μL of water was used as the collection solution to minimize the salt content in the fractions.

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The nanoliter chemistry station offers several advantages for performing digestions over bulk solution methods. Enzyme kinetics are concentration dependant, so the lower the analyte concentration, the less efficient the digestion. By drying 20 nL of CE fraction in the nano ChemStation capillary and digesting in just 1 nL of trypsin solution a much more complete digestion occurs. Furthermore, buffer salts and other contaminants are rinsed away during the drying procedure. Since contaminants are not concentrated along with the protein, there is less signal suppression during MALDI analysis. In addition, approximately 1% of the collected CE fraction volume is needed for each digestion; so there is enough remaining for repeat digestions, direct MALDI-MS analysis, or other analyses.

1.5. Conclusions

Microliter volume CE fraction collection was shown to be an effective tool for protein analysis at a subpicomole level. Hydrodynamic fraction collection combined with nanoliter chemistry techniques allows protein identification from a single CE run through molecular weight determination and peptide mapping. In the future, multiple enzyme reactions or derivatization reactions following digestion can be employed to provide further sequence information. In addition, better separation conditions need to be used to give improved separation capabilities, especially for more complex biological samples.

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1.7. Figures

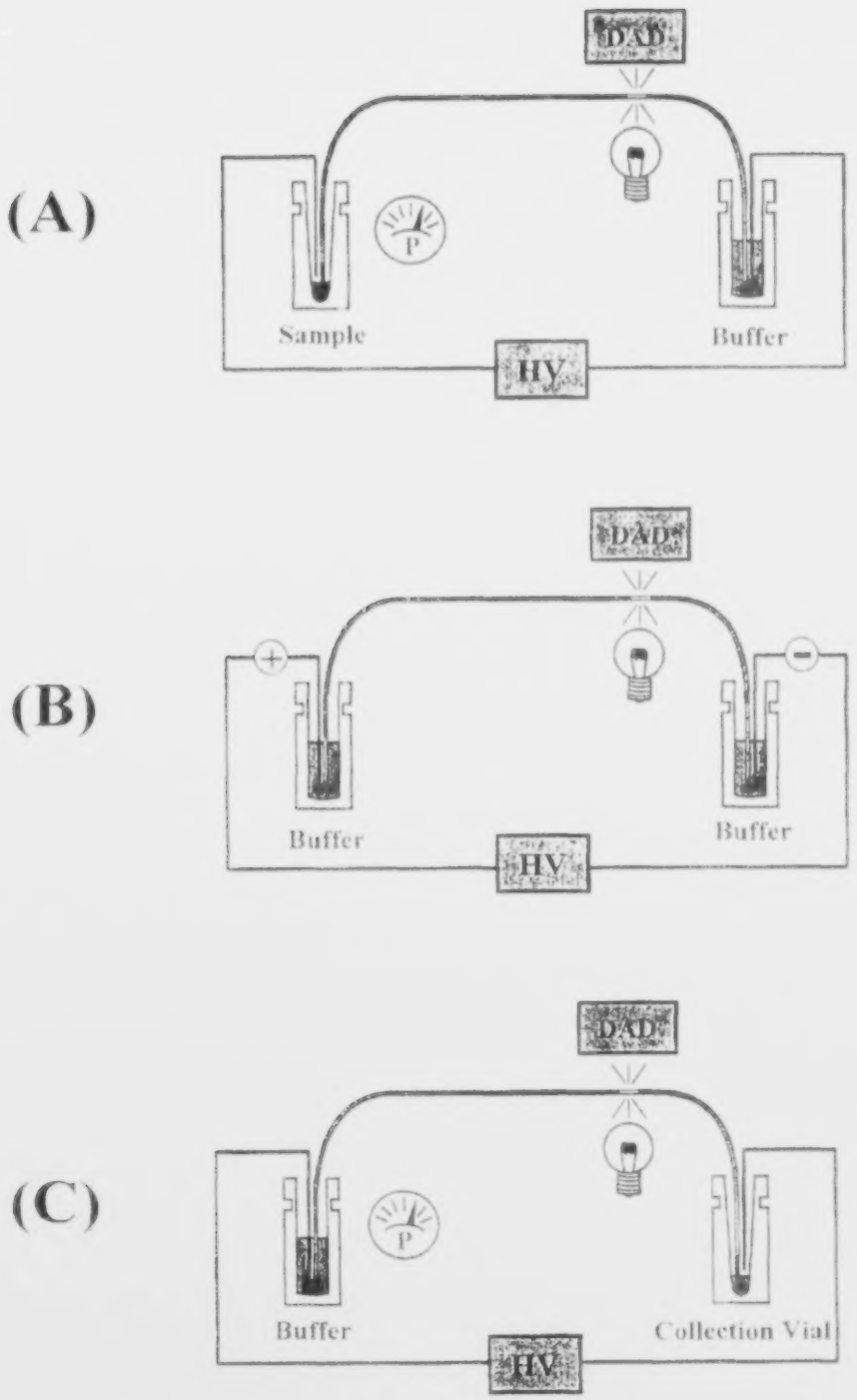

Figure 1.1. Schematic of the CE separation and fractionation setup.

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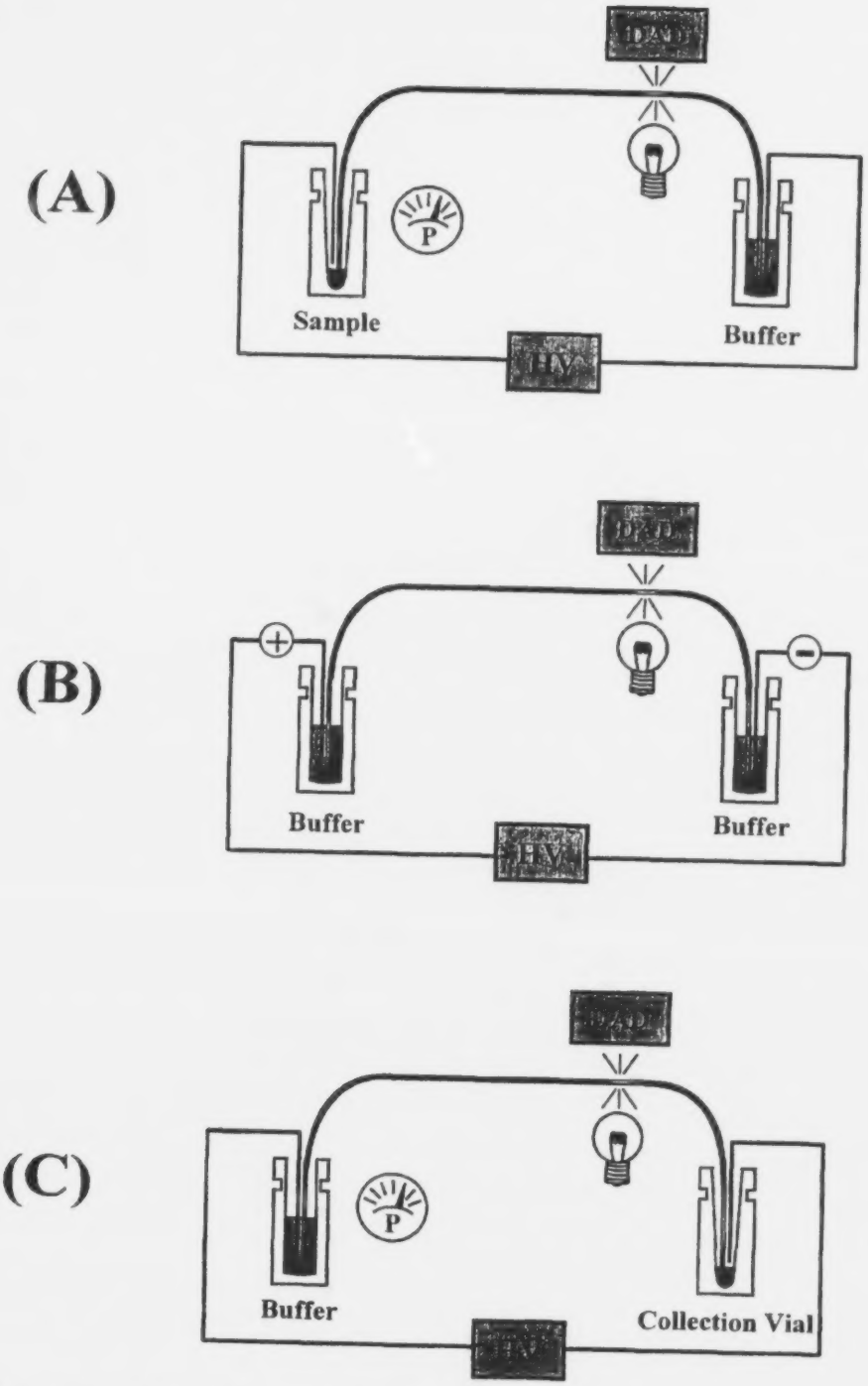

Figure 1.1. Schematic of the CE separation and fractionation setup.

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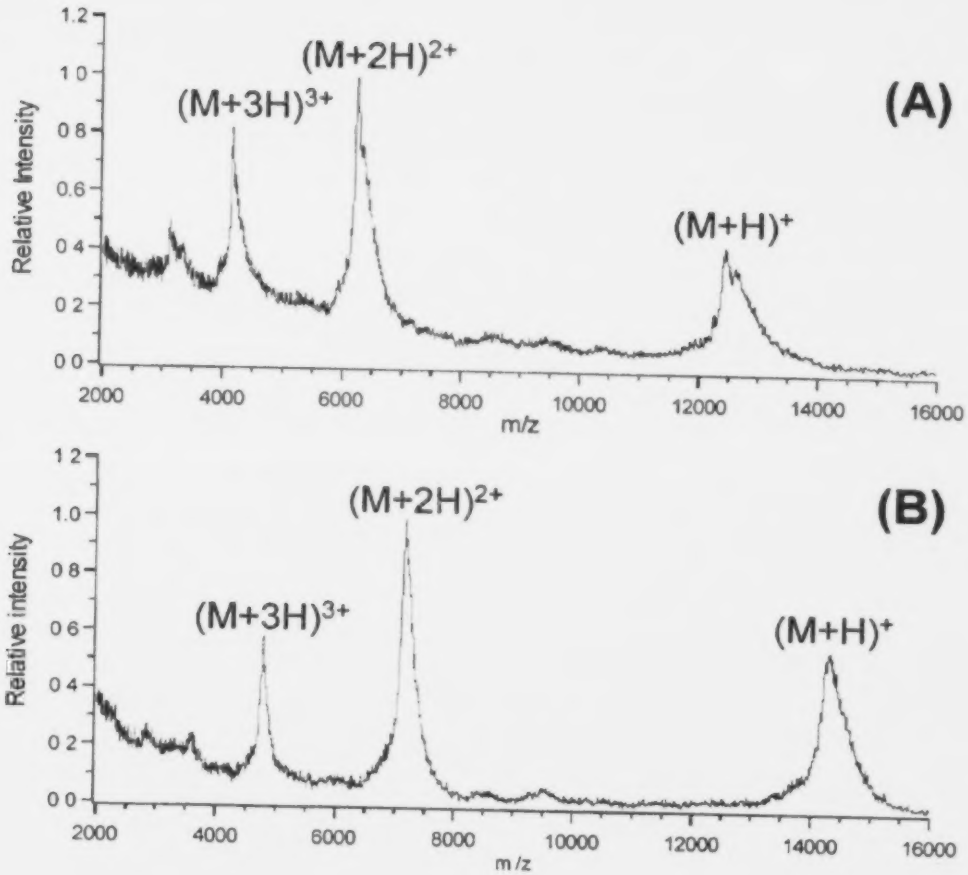

Figure 1.2. MALDI mass spectra of CE fractions containing (A) cytochrome c and (B) a-lactalbumin with 200 fmol loading on CE.

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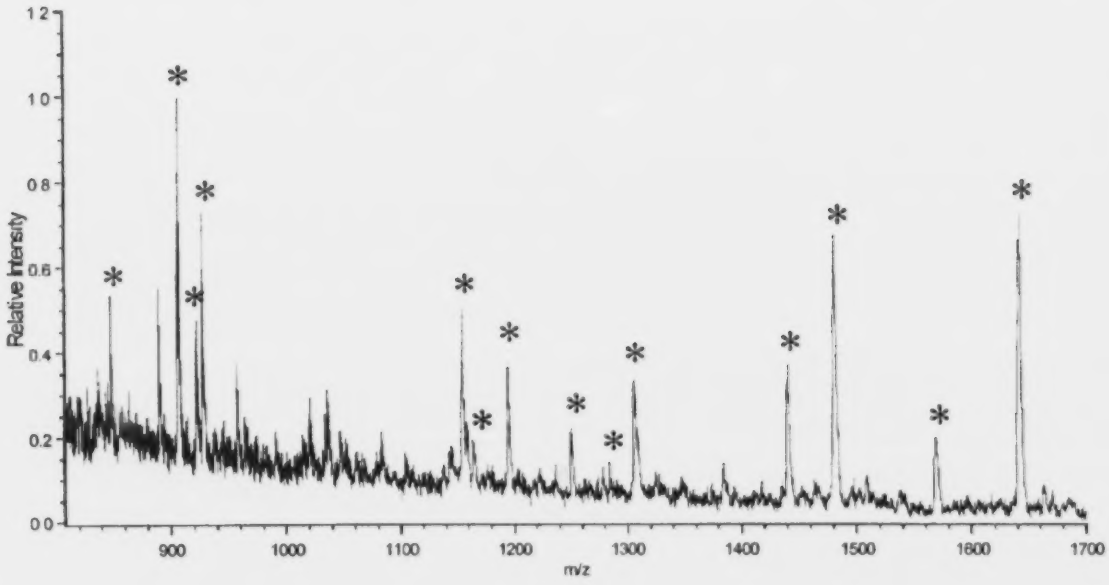

Figure 1.3. MALDI mass spectrum of the tryptic digest of a BSA fraction from CE. Marked peaks are BSA fragments.

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Part 2: Capillary Electrophoresis Using a Surfactant-Treated Capillary Coupled with Offline Matrix-Assisted Laser Desorption Ionization Mass Spectrometry for High Efficiency and Sensitivity Detection of Proteins

2.1. Summary

A method of combining capillary electrophoresis (CE) using a surfactant-modified capillary with matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) is described for protein analysis. The CE/MALDI-MS coupling is based on CE fraction collection of nanoliter volume samples in less than 5 microliters of dilute acid. This offline coupling does not require any special instrumentation and can be readily performed with commercial instruments. Protein adsorption during CE separation is prevented by coating the capillary with the surfactant didodecyldimethylammonium bromide (DDAB). This surfactant binds strongly with the capillary wall; hence it does not desorb significantly to interfere with subsequent MALDI-MS. It is shown that the use of a dilute acid for CE fraction collection is advantageous in lowering the detection limit of MALDI-MS compared to using an electrophoretic buffer. The detection limit for proteins such as cytochrome c is 23 femtomoles injected for CE, or 1.2 femtomoles spotted for MALDI-MS. This sensitivity is comparable to alternate CE/MALDI-MS coupling techniques using direct CE sample deposition on the MALDI target. In addition, the fraction collection approach has the advantage of allowing multiple reactions to be carried out on the fractioned sample. These reactions are very important in protein identification and structure analysis.

2.2. Introduction

Mass spectrometry (MS) is playing an important role for protein identification and characterization in proteomics. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) MS are commonly used. ESI generates multiply charged ions that are particularly useful for tandem MS analysis. In addition, ESI can be readily interfaced with liquid phase separations such as liquid chromatography (LC) and capillary electrophoresis (CE). MALDI, on the other hand, has a higher tolerance to impurities. It produces simple mass spectra dominated by singly and doubly charged ions. Most importantly, it generally offers higher sensitivity for protein analysis compared to ESI.

There are several reports of combining liquid separation with MALDI-MS. In addition to the continuous flow probe method for LC-MALDI coupling [1-3], other on-line approaches have also been described [4]. However, on-line LC or CE/MALDI-MS has not been widely used for real-world applications due to current technical challenges, which lead to less than desirable

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performance for peptide and protein analysis [5]. Alternatively, off-line coupling between LC and MALDI-MS can be performed by fraction collection. The offline coupling of MALDI-MS with CE, however, is less straightforward as the sample size in CE can be as low as a few nanoliters. In addition, electrical contact must be maintained during the electrophoretic separation. Methods reported for coupling CE and MALDI-MS generally involve either direct sample deposition from the CE capillary onto a MALDI target [6] or CE fraction collection followed by subsequent MALDI analysis [7].

Direct sample deposition for off-line coupling of CE with MALDI-MS results in excellent sensitivity. The sample is deposited onto targets pre-coated with MALDI matrix. The entire sample injected in CE is deposited with minimum handling. Sample deposition can be performed on a stationary MALDI plate in which the sample is spotted as droplets [8-10]. Alternatively, sample can be deposited as a trace on a moving MALDI target, such as a tape or a wheel [11-14]. The lowest detection limits reported for peptides and proteins using direct sample deposition are 50 attomoles and 2 femtomoles, respectively [12]. These numbers refer to the quantities injected into CE, and they are very close to the detection limits of the current MALDI mass spectrometers. While direct sample deposition offers low sensitivity, it does require special instrumentation alteration, which is not easily accessible by other users at present.

Fraction collection does not require special instrumental alteration, and it can be readily performed with commercial instruments. In addition, the fractionated samples can be potentially divided into smaller portions for down-stream chemical or enzymatic processing. The latter is crucial for protein identification and analysis of post-translational modifications. Multiple chemical or enzymatic reactions are necessary for achieving high amino acid coverage of a protein, thus increasing the confidence of identification or providing structural information on the modification sites. To perform fraction collection in CE, the electrophoretic separation is interrupted just before an analyte band reaches the outlet of capillary. The analyte band is then collected either hydrodynamically or electrokinetically [7]. When hydrodynamic fractionation is used, a low pressure is applied at the inlet to push the sample band off the capillary into a micro vial. However, significant band broadening is caused by the pressure-induced laminar flow during fraction collection. More importantly, the mobilities of the analytes remaining in the capillary are interrupted, and thus subsequent fraction collection is not accurate.

Alternatively, collection of the analyte band can be performed with an applied voltage. In electrokinetic fraction collection, high separation efficiency can be maintained. Nevertheless, electrokinetic fractionation is less straightforward compared to hydrodynamic fractionation. During fraction collection, an electrolyte is required to maintain the electrical current between the electrode and the capillary outlet. The electrophoretic buffer is generally used as the electrolyte. However, metal cations from the buffer, such as sodium and potassium, can drastically suppress the MALDI-MS signal of proteins. It is particularly problematic when analyzing low concentration proteins or peptides [15]. For this reason, fraction collection for

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CE/MALDI-MS is usually performed hydrodynamically; *e.g.*, in the analyses of glycoproteins [16,17]. The use of dilute acid as the fraction collection electrolyte has been reported to improve MALDI signals [18]. We illustrate herein that CE fractionation with a dilute acid can also be performed electrokinetically in an easy and reliable manner. This technique allows multiple consecutive fraction collections in one CE separation.

Another challenge in the CE/MALDI-MS analysis of proteins is the adsorption of proteins onto the capillary wall. Protein adsorption can lead to serious band broadening, sample loss and poor reproducibility. A common approach to preventing protein adsorption is to form an inner coating in the capillary [19]. The capillary surfaces can be chemically modified with polymers to form permanent coatings [20]. Alternatively, dynamic coatings can be formed through the adsorption of surfactants [21]. The coating procedures for permanent coatings are usually long and tedious. Commercial capillaries with permanent coatings are available, but they are expensive and have limited lifetimes and operating conditions. Dynamic coatings, on the other hand, are inexpensive and easy to form, *i.e.* simply by adding surfactants in the electrophoretic buffer. More importantly, superior performance has been reported in the prevention of protein adsorption with surfactant-modified capillaries. However, for coupling CE with MS, the use of surfactants has been traditionally avoided due to their incompatibility with MS detection. Surfactants form adducts with proteins and suppress sample ionization [22]. Hence CE/MALDI-MS has always been performed in permanently coated capillaries.

The use of a semi-permanent capillary wall coating formed by a two-tailed cationic surfactant, didodecyldimethylammonium bromide (DDAB), was recently reported by Melanson *et al.* [23]. Similar to other cationic surfactants, DDAB coating formation takes place by rinsing the capillary with a surfactant solution. However, the two-tail nature of DDAB results in a very strong hydrophobic effect, such that the coating remains after flushing out the DDAB solution. The coated capillary is then filled with a surfactant-free buffer, in which electrophoresis takes place. This potentially eliminates the presence of surfactant in the bulk solution inside the capillary, thus the surfactant should not interfere with the MALDI-MS measurement. The first application of surfactant-modified capillaries in CE/MALDI-MS is demonstrated and studied in this work.

2.3. Experimental

2.3.1. Apparatus.

An Agilent ^{3D}CE instrument (Palo Alto, CA) equipped with an ultraviolet-visible diode array detector was used to perform all CE separations and fraction collection. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with an inner diameter of 50 μm and an outer diameter of 365 μm were used. The total capillary length was 30.5 cm, and the length to detector

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was 22.3 cm. The capillary was thermostated at 25°C in all experiments. ChemStation software (Rev. A.06.03, Agilent) was used for data acquisition and instrument control on a Pentium based microcomputer. Detection of all proteins was performed by direct UV absorption at 192 nm. Hydrodynamic sample injection was performed at a constant pressure of 5 kPa.

Mass spectral measurements were conducted with a time-lag focusing (TLF) MALDI-TOF mass spectrometer constructed in-house [24-25]. Mass spectral data acquisition and processing was performed with Hewlett Packard supporting software HP DADA-601 (S.02.99.1101). Data was reprocessed using IGOR Pro (Version 3.13, WaveMetrics Inc., Lake Oswego, OR).

2.3.2. Chemicals.

Nanopure 18MΩ water (Barnstead) was used in the preparation of all solutions. Reagent grade orthophosphoric acid (Mallinckrodt, Paris, KY), acetic acid (Anachema, Rouses Point, NY), or formic acid (Fisher) was used to prepare the CE buffers. The pH was adjusted with reagent grade sodium hydroxide (Fisher). Hydrochloric acid used in fraction collection was purchased from Anachema. The cationic surfactant didodecyldimethylammonium bromide (DDAB, Aldrich) was used as received. MALDI matrix α-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Aldrich and purified by recrystallization from ethanol prior to use. Standard proteins cytochrome c (bovine heart, C3131), cytochrome c (horse heart, C7752), and lysozyme (chicken egg white, L6876) were used as received from Sigma.

2.3.3. CE Separation for Proteins.

Capillary pre-conditioning was performed with a high-pressure (100 kPa) rinse with NaOH for 5 min followed by distilled water for 1 min. New capillaries were used for each buffer system to avoid cross contamination. Capillary coating with DDAB was performed with a 5-min rinse (100 kPa) with 0.1 mM DDAB in water [23]. A 1-min rinse (100 kPa) with the electrophoretic buffer was used to remove the excess surfactant. Between subsequent separations, the capillary was reconditioned (100 kPa) for 2 minutes with 0.1 mM DDAB and for 1 minute with the electrophoretic buffer.

For the fraction collection solution study, samples of 0.2 $\mu g/\mu L$ horse heart cytochrome c in water were injected hydrodynamically at 5 kPa for 2.0 sec in all cases. Phosphate, acetate, or formate, 25 mM at pH 4.00, were used as the electrophoretic buffers, and the applied voltage was –6 kV. In the detection limit study, 0.2 $\mu g/\mu L$ horse heart cytochrome c was injected at 5 kPa for 1.0 sec. A phosphate buffer, 25 mM at pH 4.00, was used and the applied voltage was –6 kV.

In the separation and fraction collection of standard protein mixtures, a mixture of bovine heart cytochrome c, horse heart cytochrome c, and lysozyme (0.2 $\mu g/\mu L$ each) was injected at 5 kPa for 2.0 sec. The applied voltage in this case was –12 kV, and a 10 mM phosphate buffer at

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pH 7.00 was used. Efficiencies of all CE peaks were determined at the baseline using the tangent method.

2.3.4. CE Fraction Collection.

Collection of an analyte band at the outlet of the capillary was performed electrokinetically into a polypropylene micro insert (Agilent, Part no. 5182-0549). In the fractionation solution study, various buffers and dilute acid were placed in the insert for electrokinetic fraction collection. To minimize dilution, the volume of the fractionation solution was limited to 5 μL or less. This 5-μL volume resulted in a solution depth of *ca.* 2 mm in the micro insert. To provide electrical contact between the outlet electrode and the fractionation solution, the top of the micro insert was trimmed by 5 mm in order to raise the position of the insert closer to the electrode and capillary.

The fraction collection function programmed in the ChemStation software (Agilent) was not used in our experiment because it did not account for the vial exchange time during fraction collection. This led to erroneous fraction collection timing, particularly during short fractionations (< 30 sec) for the highly efficient protein peaks. As a result, the fraction collection was performed manually.

The timing of fraction collection is calculated assuming that the mobility of the analytes is constant during the separation. That is, the time at which an analyte band reaches the outlet of the capillary (t_{outlet}) is obtained by multiplying the recorded migration time (t_m) with the ratio of total capillary length (L_t) to the length from inlet to detector (L_d):

$$t_{outlet} = t_m \cdot \frac{L_t}{L_d} \qquad (1)$$

At t_{outlet}, the CE separation is interrupted by switching off the applied field. The electrophoretic buffer vial at the capillary outlet is removed, and the fractionation micro insert is placed at the outlet. The applied field is then resumed to perform electrokinetic fraction collection. The time length of electrokinetic fraction collection is determined by the baseline width of the peak at the capillary outlet ($W_{b,outlet}$), which is calculated from the baseline width recorded at the detector ($W_{b,detector}$):

$$W_{b,outlet} = W_{b,detector} \cdot \frac{L_t}{L_d} \qquad (2)$$

When needed, consecutive fraction collections can be performed by placing new micro inserts for fractionation. Otherwise, the electrophoretic buffer vial can be repositioned at the outlet to resume the CE separation.

2.3.5 Sample Deposition and MALDI Measurement for Protein Standards.

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A three-layer sample deposition method was used for MALDI protein analysis. This method generated reproducible mass spectra from a given sample. In this method, 1 μL of 10 mg/mL HCCA in 20% methanol/acetone v/v was first deposited on the MALDI target to form a thin layer of fine crystals. Then 0.25 μL of a saturated HCCA solution in 33% methanol/water v/v was deposited on top of the first layer and allowed to dry. Finally a 0.25 μL aliquot of the sample was spotted as a third layer and allowed to dry prior to measurement. Each analysis was repeated in triplicate, with an average of 75 laser shots summed for each mass spectrum.

2.4. Results and Discussion

2.4.1. CE Fraction Collection and MALDI-MS Measurement.

CE separations were performed in three buffer systems: 25 mM phosphate, formate and acetate, all at pH 4.00. Cytochrome c (horse heart) was used as a test protein. Fused silica capillaries were pretreated with DDAB to prevent protein adsorption onto the capillary wall [23]. Injection was performed at 5 kPa for 2.0 sec with a 0.2 μg/μL cytochrome c (horse heart) solution in water. The amount of cytochrome c injected was *ca.* 92 femtomoles in each run. The electrophorograms obtained with the different buffers are shown in Figures 2.1A-C respectively. The peak efficiencies are 600,000 plates/m in phosphate (Figure 2.1A), 300,000 plates/m in formate (Figure 2.1B) and 200,000 plates/m in acetate (Figure 2.1C). The measured efficiencies are similar to those reported previously by Melanson *et al.* [23].

The migration times of cytochrome c in all three buffers are relatively fast, from 2.0 minutes in formate to 4.4 minutes in phosphate. The fast migration times are caused by the fast electroosmotic flow (EOF) generated in the DDAB modified capillary. Most of the available coatings, either dynamic or permanent, are neutral in charge. The resulting EOF is therefore either greatly suppressed or near zero. The mobilities of analytes are solely based by their charge-to-size ratio. Slightly charged or near neutral proteins therefore migrate very slowly, resulting in long analysis times. DDAB, on the other hand, is a cationic surfactant that generates a highly cationic capillary wall coating, and thus a fast anodic EOF. Although cationic proteins migrate in the opposite direction as the anodic EOF, the magnitude of the EOF is sufficient to sweep the counter-migrating proteins to the detector. Such fast EOF is useful in carrying proteins with low or zero mobility to the detector, enabling their detection that would not be possible otherwise. The variation in the migration time among different buffers (Figure 2.1) is due to the different magnitude of EOF in various buffers [26-27].

Micro fraction collection was performed as outlined in the Experimental section. The measured peak width at baseline is *ca.* 6.5 seconds for cytochrome c in all three buffers. Taking account for the detector-to-total length ratio, this translates to an electrokinetic fraction collection

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interval of 9.0 seconds based on Equation 2. For each of the three buffer systems (Figures 2.1A-C), fraction collection was performed first in 5 μL of the electrophoretic buffer and then in 5 μL of dilute (10 ppm) HCl solution. The mass spectra of the fractions collected in 5 μL of electrophoretic buffer are presented in Figures 2.2A, 2.3A and 2.4A for phosphate, formate, and acetate respectively. The corresponding mass spectra of the cytochrome c fractions collected in HCl are shown in Figures 2.2B, 2.3B and 2.4B. As a control experiment, the mass spectrum of a 20 nM cytochrome c solution in water was also recorded. The 20 nM concentration was selected to approximate the 92 femtomoles of protein collected in 5 μL. The mass spectra of this solution represent the optimal signal intensity of cytochrome c in the absence of any signal suppression or sample loss during fraction collection. Since the MALDI measurements for the three buffers were performed on three different days, a control measurement of 20 nM cytochrome c was performed on each day. They are shown in Figures 2.2C, 2.3C and 2.4C. All spectra shown in Figures 2.2-2.4 were collected in triplicate. The spectra presented are the medians of the triplicates.

In all of the spectra recorded (Figures 2.2-2.4), three main ion peaks are detected: $(M+H)^+$, $(M+2H)^{2+}$, and $(M+3H)^{3+}$. A variation in signal intensity is observed between the different fractionation electrolytes. In all cases studied, the mass spectra of fractions collected in the electrophoretic buffer result in the lowest signal intensity (Figures 2.2A, 2.3A and 2.4A). When the same fraction collection is performed in dilute HCl, peak intensities similar to those from the 20 nM cytochrome c standard solution are observed. This confirms that MS signal suppression occurs when the electrophoretic buffers (sodium phosphate, sodium formate and sodium acetate) are used as the fraction collection electrolyte. Such MS signal suppression can be prevented by using a dilute HCl solution for fraction collection.

A potential problem in using a non-buffered electrolyte, such as dilute HCl, for electrokinetic fraction collection is electrolysis. Electrolysis at the capillary outlet can cause a pH change, which in turn may alter the protein mobility. We believe that the use of a DDAB modified capillary helped to reduce the effect of the potential electrolysis. A fast, forward-moving EOF is generated in DDAB capillaries. This EOF may help preventing the fraction collection electrolyte from entering the capillary at the outlet. In addition, a reversed polarity setting is used with a DDAB capillary, *i.e.* the anode is at the capillary outlet. If electrolysis takes place, the pH of the fraction collection electrolyte will be lowered by oxidation. Normally CE separation of proteins is performed in acidic buffers, such as pH 4 in this work. Fortunately, the ionization of proteins is less sensitive to pH changes in the acidic direction, as these proteins are close to being fully protonated at pH 4. On the other hand, normal polarity must be used for most permanently derivatized capillaries due to the suppressed EOF. Reduction takes place at the capillary outlet and increases the buffer pH. This can cause significant changes in the proteins' ionization and thus mobilities.

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Although DDAB is useful in achieving fast analysis times and allows the use of non-buffered fraction collection electrolytes, it is important to make sure that the DDAB from the capillary coating does not cause interference in MALDI-MS. The MALDI spectra in panel B of Figures 2.2-2.4 are recorded from fractions collected from DDAB-coated capillaries. The intensities of the $(M+2H)^{2+}$ peaks are 80-90% of those observed in the spectra of 20 nM cytochrome c solution (panel C in Figures 2.2-2.4). The small difference in intensity may reflect the different amount of cytochrome c used, 92 femtomoles from the CE fraction and 100 femtomoles from 20 nM solution. In addition, sample handling during fraction collection, such as solution transfer between vials, can also result in sample loss. Overall, significant interference caused by DDAB is not evident. The first coupling of CE and MALDI-MS with the use of a surfactant modified capillary is therefore successfully demonstrated. The use of permanently derivatized capillaries is no longer the only choice for CE/MALDI-MS.

2.4.2 Detection limit

To determine the detection limit of our MALDI-MS measurements on the CE fractions with 10 ppm HCl, we performed CE/MALDI-MS with different amounts of cytochrome c (horse heart) injected. The separation was performed in phosphate buffer (25 mM) at pH 4.00, and fraction collection was performed as previously described at –6 kV for 9.0 seconds. The signal-to-noise ratio (S/N) of the $(M+2H)^{2+}$ peak was recorded for each sample in four replicates. The lowest amount of cytochrome c (horse heart) injected that resulted in an MS signal of $S/N \geq 3$ in all four replicates is 23 femtomoles, with S/N ranging from 6 to 60. The wide range of signals observed from the replicate experiments indicates that this amount is close to the detection limit. Indeed, any lower amount of cytochrome c resulted in signals below the detection limit in at least one of the four replicates. The lowest detection limit reported in the literature for CE/MALDI-MS is 2 femtomoles for proteins injected in CE [12]. Our detection limit is about 10 times higher. Unlike the direct sample deposition used in reference 12, not all of the protein collected in each fraction is used for MALDI-MS in our case. Out of the 5 μL in each fraction, only 0.25 μL is actually spotted onto the MALDI probe for each measurement. Hence the actual detection limit of MALDI-MS is about 1.2 femtomoles. The detection limit of our laboratory-built MALDI instrument is around 1 femtomole for proteins such as cytochrome c [28]. In other words, the MALDI-MS detection limit is not affected by the CE separation and fraction collection.

Because only 0.25 μL is consumed for each MALDI-MS measurement, we can, in principle, perform 20 measurements for each 5 μL CE fraction. Alternatively, the 5-μL fraction can be divided into several portions for various sample treatments such as enzymatic digestions and chemical reactions. We are currently developing and applying a nanoliter-volume sample

handling technique to carry out post-separation sample treatments for protein identification and structure analysis.

When post-separation sample treatment is not required, we can reduce the amount of fractionation electrolyte to less than 5 μL in order to lower the detection limit. The smallest amount of fractionation electrolyte used was 2 μL. In theory this can lower the detection limit from 23 femtomoles to 9 femtomoles, which is closer to the low detection limit record of 2 femtomoles reported [12]. Nevertheless, the 2-μL fractionation electrolyte is so small that it occasionally does not result in a successful collection, presumably when it fails to maintain electrical contact between the electrode and the capillary outlet. Care must be exercised when using such small volumes of fractionation electrolyte.

An alternate approach has been attempted to increase the sensitivity, namely by concentrating the collected fraction prior to MALDI measurements. A 5-μL fraction was transferred to a siliconized polypropylene vial (600 μL) and evaporated down to 1 μL by vacuum centrifugation. Unfortunately, such sample concentration was not successful as the protein was presumably lost due to adsorption onto the container. As a result, the 'concentrated' samples actually resulted in lower MALDI signals. Other sample concentration techniques, such as the use of reverse phase particles [29], will be investigated in the future.

2.4.3. Accuracy of Fraction Collection Timing

Since the separation efficiency in this CE method is very high, a suitable means of collecting fractions for only a few seconds is required. The timing of our electrophoretic fraction collection is based on the extrapolation of the migration time recorded by an online detector. Errors may arise from any changes in analyte mobility during electrophoresis and fraction collection. Electrolysis can take place at the electrode to alter the pH, protein mobility and EOF. Additional band broadening or siphoning during vial transfers, as well as the uncertainty of the voltage control during ramping, can also give rise to erroneous timing during fraction collection.

To evaluate the accuracy of our fraction collection, we performed multiple consecutive fraction collections within one CE separation. The CE separation of a mixture of three proteins, cytochrome c (bovine heart), cytochrome c (horse heart) and lysozyme (chicken egg white), was performed at –12 kV in a pH 7.0 phosphate buffer (10 mM). The proteins and the experimental conditions were optimized to yield three closely resolved peaks (Figure 2.5). Poor accuracy in the fractionation timing would result in collection of unintended components, which would be reflected in the subsequent MALDI mass spectra. Based on the calculation from Equations (1) and (2), collection of the three protein peaks was performed as three consecutive fractions with intervals of 10-12 seconds at -12 kV. MALDI mass spectra were recorded for the fractions. Initially the MALDI signals appeared to be weaker than that recorded previously when the

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fractionation voltage was -6 kV. A similar observation has been reported by others [30]. It is caused by a loss of analytes when high voltage (-12 kV) is used in electrokinetic fraction collection. Sample binding or interaction with the outlet electrode occurs, resulting in incomplete collection. The problem can be solved by simply reducing the fractionation voltage and adjusting the collection time duration proportionally [7]. In this case, fractionation was performed at half the voltage (- 6 kV) for twice the time duration (18 seconds). The intensity of the MS signal improved significantly with the reduced fractionation voltage.

The MALDI mass spectra of the cytochrome c (bovine heart), cytochrome c (horse heart), and lysozyme CE fractions are presented in panels A-C respectively of Figure 2.6. In general, the major peaks observed in each mass spectrum correspond to the protein expected. In the first fraction (Figure 2.6A), only signals from cytochrome c (bovine heart) are observed. In the second fraction (Figure 2.6B), small quantities of lysozyme, $(M+2H)^{2+}$, is observed. Likewise, small signals of cytochrome c (bovine heart) and cytochrome c (horse heart) are detected in the third fraction (Figure 2.6C). These results show that multiple consecutive fraction collections can be done within a short collection time and collection of the intended component is evident in the MALDI-MS results. Using dilute HCl as fraction collection electrolyte did not lead to any significant error in the collection timing. Thus the mobility of the proteins is not altered significantly by electrolysis during fractionation. Overall, our fraction collection is performed in a precisely controlled manner.

2.5. Conclusions

Micro fraction collection in CE offers simple offline coupling with MALDI-MS. The MALDI-MS detection limit is not significantly affected when analyzing proteins in CE fractions. The timing of the fraction collection is well controlled using electrokinetic fractionation. Collection of multiple consecutive fractions in one CE separation within a short time window was demonstrated. The use of capillaries treated with the two-tailed surfactant DDAB prevents adsorption of proteins and provides a fast EOF for short separations. Unlike other surfactants, DDAB forms a semi-permanent wall coating, and thus is compatible with subsequent MALDI-MS measurements. The choice of electrolyte for CE fraction collection is critical. Ions from buffers tend to interfere with the MS signals, so dilute acid (10 ppm HCl) should be used instead. It is shown that the use of dilute acid does not alter separation efficiency and fraction collection accuracy. To further develop this CE/MALDI-MS method into a powerful tool for proteomics application, future work will mainly focus on developing a means of performing chemical and enzymatic reactions on individual fractions for protein identification and structure analysis. Initial work presented in part 1 of this report demonstrated that it was possible to combine the nanoliter chemistry station with CE fractionation using a covalently modified capillary. Future work will combine this nanoliter chemistry station with the new separation and fractionation method described in this work.

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2.6. References

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2.7. Figures

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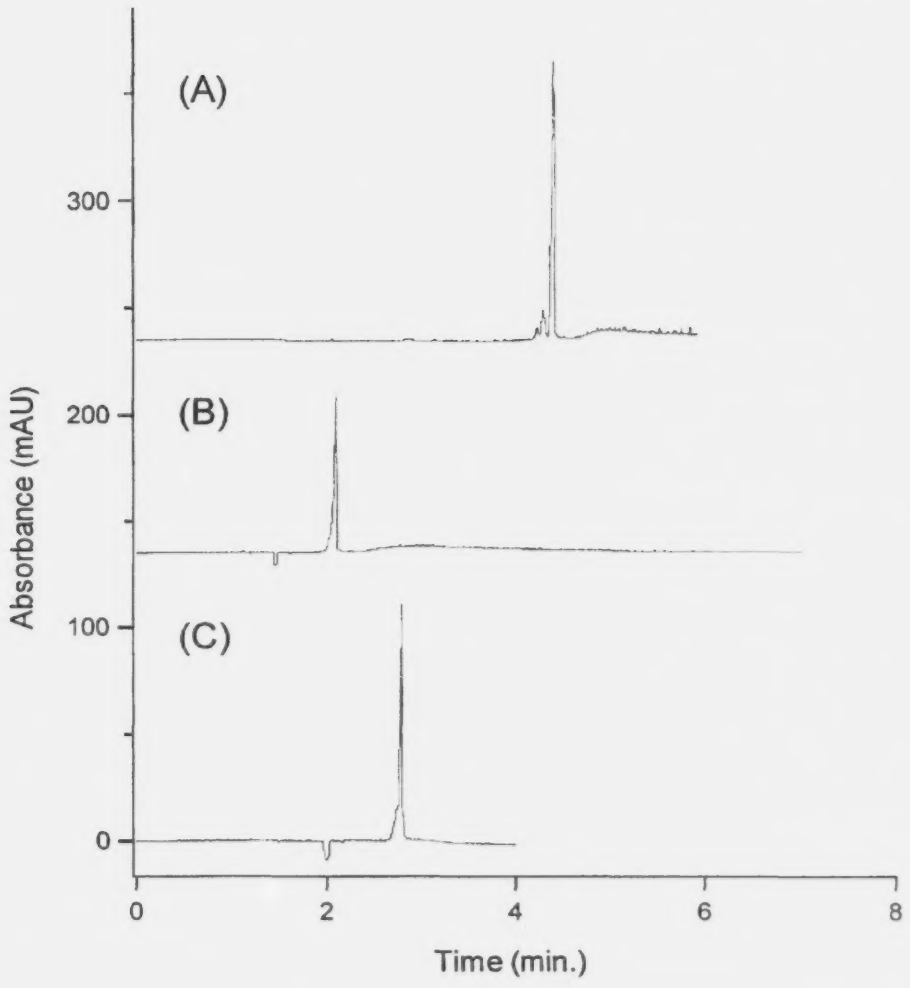

Figure 2.1. CE electrophorograms of horse heart cytochrome c in (A) phosphate, (B) formate, and (C) acetate. Experiment conditions: capillary lengths, 22.3 cm (to detector) and 30.5 cm (total); separation voltage, –6 kV. The EOF is determined by injection of acetonitrile (A) or water (B-C).

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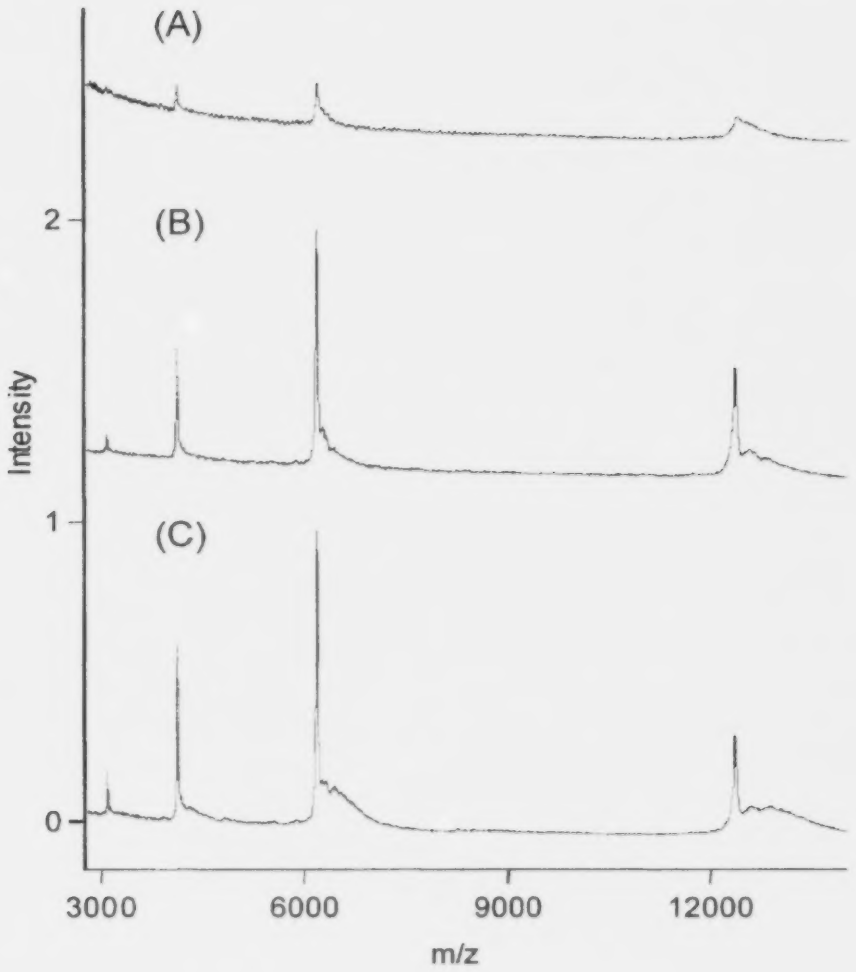

Figure 2.2. MALDI mass spectra of cytochrome c from (A) CE fraction collected in electrophoretic buffer, (B) CE fraction collected in 10 ppm HCl, and (C) a 20 nM cytochrome c solution. CE separation was performed in 25 mM sodium phosphate at pH 4.0.

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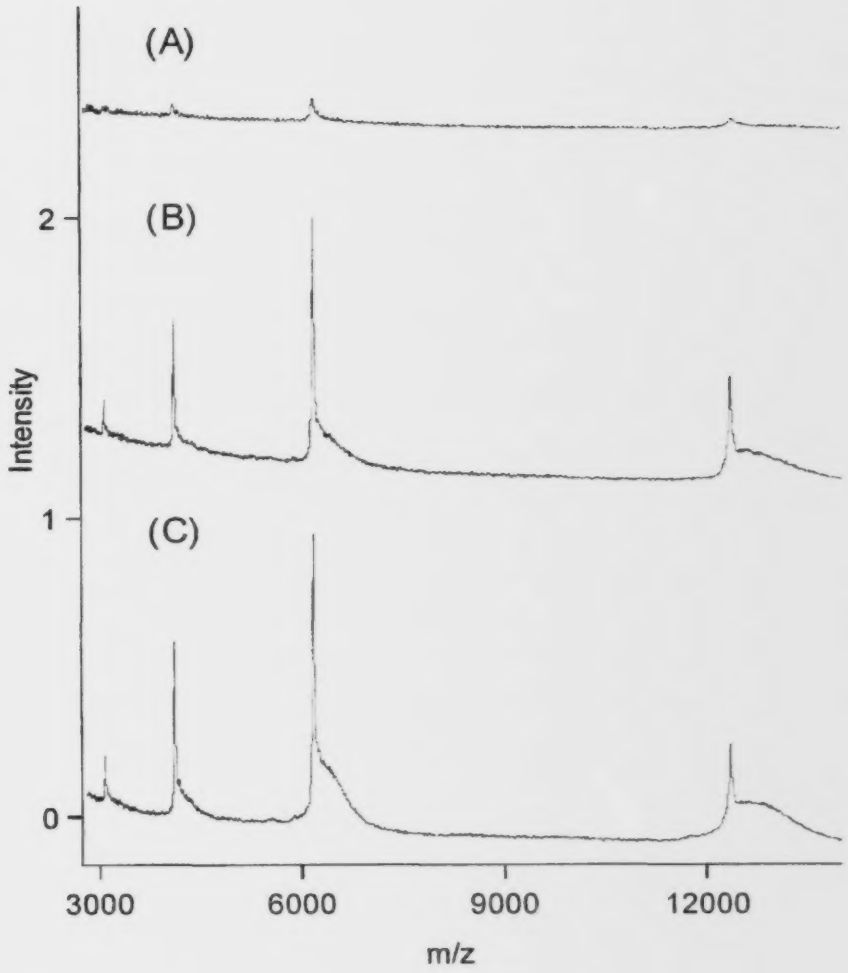

Figure 2.3. MALDI mass spectra of cytochrome c from (A) CE fraction collected in electrophoretic buffer, (B) CE fraction collected in 10 ppm HCl, and (C) a 20 nM cytochrome c solution. CE separation was performed in 25 mM sodium formate at pH 4.0.

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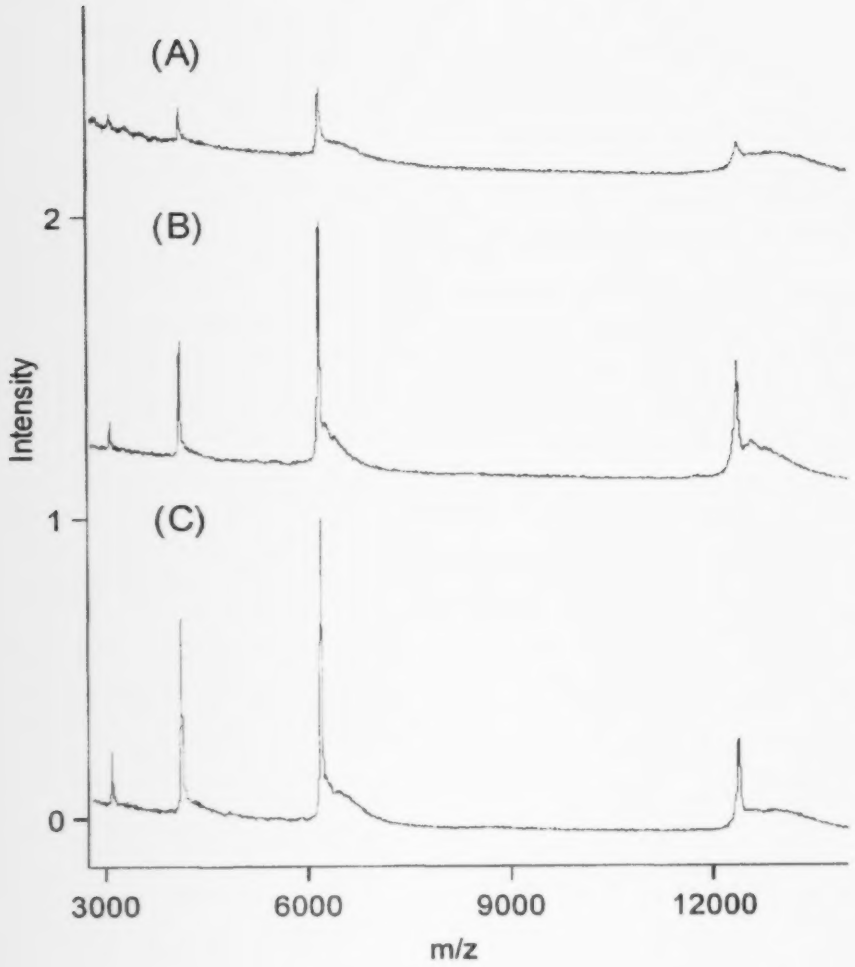

Figure 2.4. MALDI mass spectra of cytochrome c from (A) CE fraction collected in electrophoretic buffer, (B) CE fraction collected in 10 ppm HCl, and (C) a 20 nM cytochrome c solution. CE separation was performed in 25 mM sodium acetate at pH 4.0.

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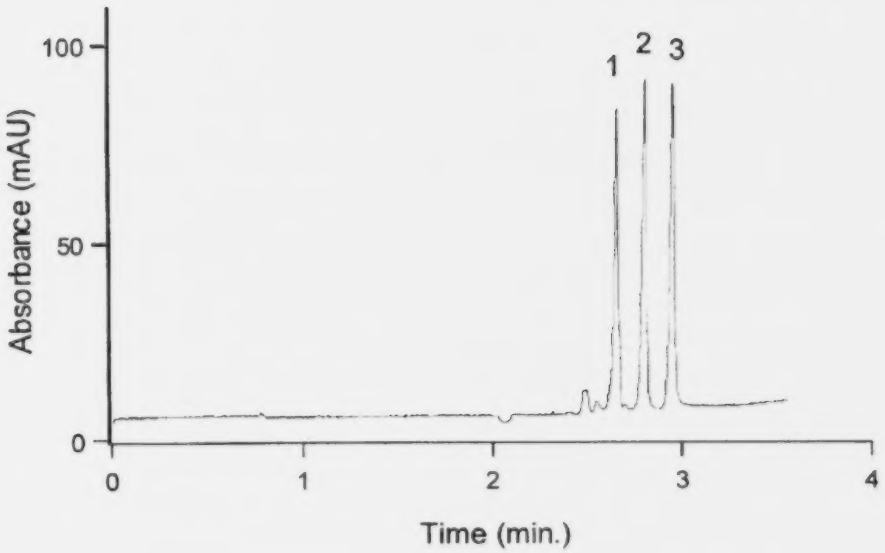

Figure 2.5. CE separation of a mixture of bovine heart cytochrome c (1), horse heart cytochrome c (2), and lysozyme (3). Experiment conditions: buffer, 10 mM phosphate at pH 7.0; voltage, −12kV; other conditions as stated in Figure 2.1.

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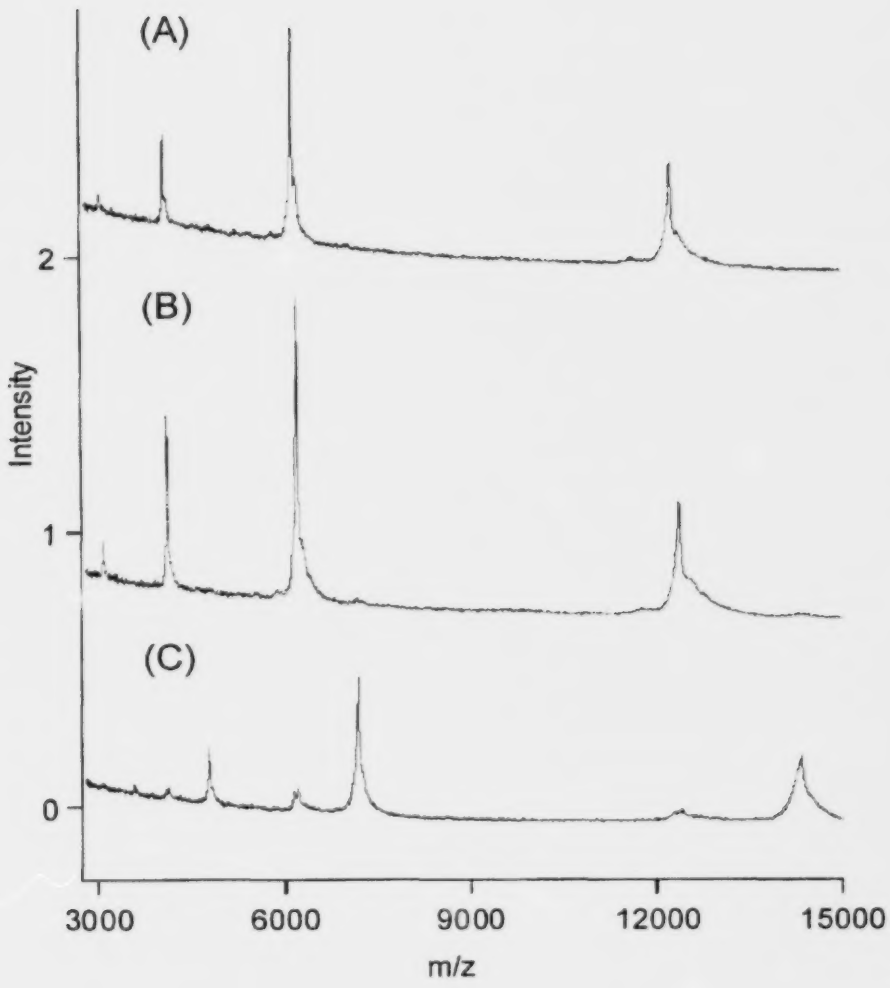

Figure 2.6. MALDI mass spectra of CE fractions for peaks 1, 2 and 3 from Figure 5 (A-C, respectively). The lower case letters refer to the peaks identified for bovine heart cytochrome c (a), horse heart cytochrome c (b), and lysozyme (c).

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Part 3: Capillary Zone Electrophoresis Combined with Matrix-Assisted Laser Desorption Ionization Mass spectrometry for the Analysis of Complex Protein Mixtures from Cell Lysates of *Escherichia coli*

3.1. Summary

Capillary zone electrophoresis (CZE) combined with matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) is applied for the separation and detection of a complex protein mixture from the cell lysate of *Escherichia coli*. Prevention of protein interaction with the capillary wall is achieved by a semi-permanent coating of the cationic surfactant didodecyldimethylammonium bromide (DDAB). Microliter volume fraction collection is performed on CZE to facilitate off-line coupling with MALDI-MS. To further improve the separation and detection, liquid chromatography (LC) is employed for pre-separation of the crude mixture, followed by CZE of individual LC fractions. The performances of CZE/MALDI and LC-CZE/MALDI are evaluated and compared for the detectability of proteins from the *E. coli* cell lysate. It is shown that effective separation is necessary to reduce ion suppression in MALDI-MS of a protein mixture, thus allowing detection of a greater number of proteins.

3.2. Introduction

Capillary electrophoresis (CE) is widely recognized as a separation technique with high separation efficiency and fast analysis time. Applications of CE range from the analysis of small inorganic ions to large biological molecules [1]. Its application to DNA sequencing was one of the most influential advances in the human genome project [2-5]. CE Instruments based on an array of 96 capillaries are commercially available for fully automated DNA sequencing [2]. In post-genome research, researchers are focusing on the study of the proteome, or the proteins expressed by the genome of a cell. At present, proteome display is most commonly performed with gel electrophoresis. While gel electrophoresis is very powerful, it is a slow and laborious technique. Research is therefore driven towards the development of alternative techniques with high sample throughput and full automation capability.

Capillary zone electrophoresis (CZE) is one of the promising candidates. CZE offers multiplex capability, fast analysis time, and high separation efficiency. Nevertheless, the application of CZE to protein separation is plagued by the adsorption of proteins onto the inner capillary surfaces. CZE is performed in fused silica capillaries. The silanols on the capillary surface ionize, making the wall anionic, and oppositely charged proteins are electrostatically attracted onto the capillary wall. Protein adsorption leads to serious band broadening, sample

loss and poor reproducibility. A number of approaches have been reported for the prevention of analyte interaction with the capillary wall in CZE [6]. Most approaches involve forming a coating on the inner wall to inhibit protein-capillary interactions. These coatings include permanent covalently modified coatings based on polymers and dynamic coatings formed by surfactants or polymers [7,8]. Excellent performance has been observed from both permanent and dynamic coatings. Quantitative sample recovery (near 100%) and excellent efficiency (in the order of 10^6 plates/m) have been reported. However, all successful CZE separations of proteins have been demonstrated with synthetic mixtures of standard proteins, such as lysozyme, α-chymotrypsinogen A and cytochrome *c*. Only a few applications of CZE in complex protein mixtures from real world samples have been reported in the literature. They were limited to samples containing a restricted number of proteins, such as recombinant human growth hormone in cell extracts [9] and glycoproteins in urine samples [10]. In this work, we report our efforts in developing and evaluating a CZE technique for the analysis of complex protein mixtures extracted from *Escherichia coli* cells. Such cell lysates contain proteins that exhibit a wide range of p*I*s, molecular weights, and hydrophobicities in the presence of background substances including salts, buffer, and other non-protein molecules.

One of the major advantages of CZE in proteome analysis is its ability to combine with mass spectrometry for protein mass analysis and identification. MS has limited utility when analyzing proteins in a complex mixture. The abundant proteins will significantly suppress the signals from low concentration proteins. A typical mass spectrum of proteins from a cell extract is dominated by a few abundant proteins, even though hundreds of proteins are present at concentrations above the MS detection limit. The signals of many proteins are lost as a result of ion suppression. It is thus often necessary to perform a separation that isolates the various components in the mixture prior to mass spectral measurements. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) is used in this work for analysis of the separated components from CZE. The combination of CZE with MALDI-MS is less common than with electrospray ionization (ESI) MS, which offers direct on-line coupling for minimal sample handling. Nevertheless, MALDI offers high sensitivity (typically in the nM range) for proteins compared to ESI (typically in the μM range). Off-line coupling of CZE and MALDI-MS can be performed either by fraction collection into a micro-vial containing a solution, or by direct deposition onto a MALDI sample target [11]. In this work, the micro fraction collection approach is chosen, as it does not require any modification to the commercial CE instrument. In addition, fraction collection provides the flexibility of allowing the collected sample to be divided for multiple downstream treatments, such as enzymatic digestion and other chemical reactions, which are extremely useful for protein identification based on mass spectrometric peptide mass mapping and fragment ion spectra of peptides.

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3.3. Experimental

3.3.1. Apparatus

All CZE separations and fraction collections were performed with an Agilent ^{3D}CE instrument (Palo Alto, CA) equipped with an ultraviolet-visible diode array detector. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with an inner diameter of 50 μm, an outer diameter of 365 μm, and a total length of 30.5 cm (22.3 cm to the detector) were used. The applied voltage used during electrophoresis was –6 kV. The capillary was thermostated at 25°C in all experiments. Data acquisition and instrument control was performed using ChemStation software (Rev. A.06.03, Agilent) on a Pentium based microcomputer. Direct UV absorbance detection was performed at 192 nm. All CZE sample injections were performed hydrodynamically at a constant pressure of 50 mbar (5 kPa).

The undesirable interaction of proteins with the capillary was prevented by coating the capillary with the cationic surfactant didodecyldimethylammonium bromide (DDAB) [12]. The coating was readily formed by rinsing the capillary with a DDAB solution. Thus the laborious procedure of permanent coating formation was avoided. Unlike other surfactant coatings, the DDAB coating is semi-permanent; *i.e.* it remains in place even after flushing out the DDAB solution. Hence CZE can be performed in a surfactant-free buffer, which eliminates the potential interference of surfactants in MS measurement [13].

Mass spectral measurements were conducted with a time-lag focusing MALDI-TOF mass spectrometer constructed in-house [14,15]. Mass spectral data acquisition and processing was performed with Hewlett Packard supporting software. Data was reprocessed using IGOR Pro (Ver. 3.13, Wavemetrics Inc., Lake Oswego, OR) software.

Sample pre-separation by preparative LC was performed on a laboratory-packed column. Reverse-phase C8 particles with 20-30 μm diameters (208TP2030, Vydac) were slurry-packed into a Pasteur pipette. Approximately 0.5 g of C8 particles was used to yield a chromatographic bed of *ca.* 0.5 cm by 4.5 cm. A small piece of glass wool was used as an outlet frit.

3.3.2. Chemicals

All solutions were prepared in Nanopure 18MΩ water (Barnstead). Buffers were prepared from reagent grade orthophosphoric acid (Mallinckrodt, Paris, KY), and the pH was adjusted with reagent grade sodium hydroxide (Fisher). Hydrochloric acid used in fraction collection was purchased from Anachema. Spectrophotometric grade trifluoroacetic acid (Aldrich) and HPLC grade acetonitrile (Fisher) were used as received. DDAB was used as received from Aldrich. MALDI matrix α-cyano-4-hydroxycinnamic acid (HCCA; Aldrich) was purified by recrystallization from ethanol prior to use.

Lyophilized *Escherichia coli* cells (ATCC 9637, strain W) were obtained from Sigma-Aldrich. Extraction of proteins from the *E. coli* cells was performed in 0.1 % (v/v) aqueous

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TFA. The supernatant was isolated by centrifugation and concentrated by evaporation (Speed-Vac, Eppendorf Vacufuge™) prior to CZE injection.

3.3.3. Protein Separation by CZE

The protein sample was extracted from 125 mg of lyophilized cells in 7 mL of 0.1 % v/v TFA. Such acid extractions are selective toward low molecular weight proteins (≤15 000 Da) [16]. The supernatant was first evaporated to *ca.* 700 µL (10-fold), and then further concentrated with a 3000 Da molecular weight cutoff filter (Microcon YM-3, Millipore Corp., Bedford, MA). The final volume of the protein solution was 125 µL.

New capillaries were used for each protein sample to avoid possible cross contamination. Prior to separation, each capillary was preconditioned with a high-pressure (100 kPa) flush of NaOH for 5 min and distilled water for 1 min. Initial treatment of the capillary surface with DDAB was performed by a 5-min rinse (100 kPa) with 0.1 mM DDAB in water, followed by a 1-min rinse (100 kPa) with the electrophoretic buffer [12]. A pH 4.0 sodium phosphate buffer (25 mM) was used in all experiments. Between subsequent separations, the capillary was rinsed (100 kPa) with 0.1 mM DDAB for 2 min and electrophoretic buffer for 1 min. Sample injection was performed hydrodynamically at 5 kPa for 1.0 to 10.0 seconds, depending on the sample concentration.

3.3.4. CZE Fraction Collection

Collection of an analyte band at the outlet of the capillary was performed electrokinetically into polypropylene micro inserts (Agilent, Part no. 5182-0549). The fraction collection timing was calculated based on the analyte's migration time and the ratio between the total length and length to detector of the capillary. A dilute (10 ppm) solution of HCl was placed in the micro insert to maintain electrical contact between the capillary and electrode. The use of HCl solution instead of electrophoretic buffer minimized the amount of sodium ions in the fraction [17]. When acquiring MALDI mass spectra of low concentration (nanomolar) proteins, the presence of sodium ions leads to adduct formation, which in turn results in broad MS peaks with lower signal-to-noise ratios [11,18].

To reduce dilution during fraction collection, the volume of dilute HCl used was minimized. The smallest amount required was 2 μL, which resulted in a solution depth of ~0.75 mm at the bottom of the micro-insert. However, the 2-μL volume did not result in successful collection in every case, so a 4-μL volume was used in most cases to ensure electrical contact between the electrode and the capillary outlet. The top 5 mm of the micro-insert was trimmed off to raise the position of the insert and thus bring the electrode and capillary outlet closer to the bottom of the micro-insert.

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3.3.5. Pre-separation by LC

A preparative-scale crude separation was performed by liquid chromatography in some cases prior to CZE. Proteins were extracted from 100 mg of *E. coli* in TFA. The supernatant was concentrated by evaporation and then loaded directly onto the column (0.5 cm x 4.5 cm). A washing step was performed with 1.0 mL of 0.05% (v/v) aqueous TFA. Then the sample was eluted with consecutive 0.5 mL portions of mobile phase with increasing acetonitrile content (v/v): 5 portions of 5%, 5 portions of 10%, 3 portions of 20%, 2 portions of 30%, 2 portions of 40%, 1 portion of 50% and 1 portion of 60% in 0.05% (v/v) TFA. Fraction collection was performed for each portion, with a total of nineteen fractions in all.

3.3.6. MALDI Sample Preparation and MS Analysis

A two-layer sample deposition procedure was used for proteins extracted from *E. coli* [19]. The MALDI target was prepared by depositing 1 μL of the first-layer matrix solution (10 mg/mL HCCA in 20% methanol/acetone v/v) and allowing it to dry and form a thin layer of fine crystals. The sample was then mixed 1:1 with FPW matrix solution (1:2:3 formic acid/isopropanol/water saturated with HCCA), and 0.25 μL of the mixture was deposited on top of the first layer. Experience in our group has shown that FPW matrix increases the MS sensitivity of proteins extracted from bacterial cells. The use of high concentration acids has also been reported by others to improve MS signals in the presence of salts [20].

Prior to MS measurement, the dried sample spot was washed twice. A drop of water (1 μL) was deposited over the sample spot and then removed with a Kimwipe after 3 seconds. This washing step removed salts present in the sample, minimizing salt-protein adduct formation and in turn improving the MS signal.

3.4. Results and Discussion

3.4.1. CZE and MALDI MS

The initial results on the CZE separation of *E. coli* cell extracts are presented in Figure 3.1A. The injection time was 1.0 second. The UV absorbance signals are observed from 2 to 8 minutes as partially resolved peaks and shoulders. The acid (TFA) extraction used in this experiment is selective towards proteins with neutral or alkaline isoelectric points. These basic proteins are cationically charged under the CZE conditions (pH 4.0); hence their electrophoretic mobility is cathodic. Capillaries treated with DDAB have cationic inner walls, and a fast anodic electroosmotic flow (EOF) is generated. As a result, the cationic proteins migrate opposite to the EOF. The magnitude of the reversed EOF is large (6.3×10^{-4} cm^2/Vs), and it is sufficient to carry the counter-migrating proteins toward the anode. Hence reversed polarity is used and the migration order is: anions, neutral molecules, and finally cations.

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The first peak observed at 2.5 minutes in Figure 2.1A is trifluoroacetate. Trifluoroacetate is a fast moving anion, thus it arrives at the detector first under reversed polarity. The triangular peak shape observed, due to peak fronting, is characteristic of electrodispersion. Electrodispersion results from differences in mobility between the analyte (trifluoroacetate) and the background co-ion (phosphate) [21]. Besides the TFA peak, strong signals are mostly detected between 3 and 5 minutes. Injection of a neutral marker (acetonitrile) resulted in a migration time of 3 minutes. This result indicates that the signals observed around this region are from neutral or slightly charged molecules. The signals beyond 3 minutes are from cationic molecules, with faster cations detected at later migration times.

The detection of neutral or slightly charged molecules is often difficult in coated capillaries. Most capillary inner coatings, both dynamic and permanent, are neutral in surface charge. The resultant EOF is either greatly suppressed or near zero. Under such conditions, the migration of analytes is solely determined by their charge-to-size ratio. Near-neutral proteins would migrate very slowly, resulting in long analysis times. DDAB coating, on the other hand, generates a fast anodic EOF. This fast EOF carries the slow migrating proteins to the detector, greatly reducing the analysis time.

The peak efficiency observed in Figure 3.1 is rather poor. Previously reported peak efficiencies from DDAB treated capillaries were greater than 500,000 plates/m [12]. There, the analytes were standard proteins such as α-chymotrypsinogen A, ribonuclease A, cytochrome *c* and lysozyme. The separation was performed at optimal conditions for minimized band broadening. These results could be readily reproduced with our instrument using the same conditions. However, in this work the proteins are from cell lysates of *E. coli*. Proteins of various MWs, p*I*s and concentrations are extracted in TFA. Hundreds of protein species together with salts and TFA are expected to be present in the sample. Thus band broadening cannot be controlled easily. The combination of significant band broadening and incomplete resolution result in the broad, overlapping peaks observed in Figure 3.1A.

To investigate the degree of separation achieved under the conditions used in Figure 3.1A, we performed MALDI-MS measurements on the fractions collected at the capillary outlet. Unfortunately, a useful MS signal was not detected from any of the fractions. With standard proteins such as cytochrome c, our CZE fractionation procedure allows the MALDI detection of less than 25 fmol of protein [17, see part 2 of this report]. It is thus likely that the individual fractions contain very little protein. We attempted to increase the MALDI signals by reducing the dilution at fractionation, *i.e.* by using less HCl solution (2 μL vs. 4 μL). With this change, however, we still could not record any protein signals. Since the cell extract sample could not be further concentrated without precipitation, another option was to increase the analyte loading to CZE by increasing the injection volume. In this case, we increased the injection volume from 2.8 nL (1.0 second injection) to 14.1 nL (5.0 second injection). The resulting electrophorogram is shown in Figure 3.1B.

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Figure 3.1B shows that there is an overall increase in the UV intensity from the larger sample loading. However, the efficiency and resolution has decreased as a result of the longer injection time. A 5.0 second injection time corresponds to an injection length of 7.2 mm. To avoid injection band broadening, injection length should be limited to 1 mm in this case [22]. However, the increased injection was necessary to deliver a sufficient amount of protein for subsequent MS measurement. Six fractions were collected as indicated in Figure 3.1B and their corresponding MALDI mass spectra are shown in Figures 3.2A-F. The spectrum of the original sample injected is included as part O.

As Figure 3.1B shows, fractions A to C are from the migration region of neutral or slightly charged proteins (3 to 5 minutes), whereas fractions D to F are from the region of the cationic proteins (5 to 10 minutes). One major protein (2579 Da) is observed from fractions A to C. The majority of proteins are found in fractions D to F, which correspond to the CZE migration region of cationic proteins. Different proteins are found in fractions D and E: 10534 Da in fraction D, and 8225 Da in fraction F. In addition, variations in the relative peak intensity among fractions are observed. These differences indicate variations in the protein compositions between the fractions, thus separation of proteins is evident within this region, namely from 5 to 8 minutes on the electrophorogram (Figure 3.1B).

Eight proteins are identified from Figures 3.2A-F and they are listed in Table 3.1. Protein identification was based on protein mass comparison (0.05% mass accuracy) with the SWISS-PROT and TrEMBL *E. coli* proteome databases. The number of proteins identified from the CZE fractions (Column 1 of Table 3.1) is the same as those identified from MALDI spectra of the original *E. coli* extract (Figure 3O). Detection of new MALDI-MS signals is not observed after CZE separation. CZE alone is unable to isolate the less abundant proteins and reduce the signal suppression during MALDI-MS measurement. The protein mixture in the raw *E. coli* lysate was too complex for the 10-minute CZE separation. We subsequently investigated the use of liquid chromatography as a sample cleanup or pre-separation method for the raw extract prior to CZE.

2.4.2 Pre-separation with Preparative LC

Separation by electrophoresis is based on the differential charge-to-size ratio of molecules. An effective pre-separation should involve an orthogonal separation mechanism. Reverse-phase liquid chromatography has been previously employed in the separation of *E. coli* proteins [23]. Such separation is based on the hydrophobic interaction of proteins with the stationary phase. The elution order of proteins follows the increasing order of their molecular weight. With gradient elution, the separation takes approximately one hour. For our application, a fast and simple sample cleanup, with large sample loading capacity, was desired. Instead of an analytical column, a laboratory-packed preparative column (0.5 cm x 4.5 cm) of C8 particles was used.

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Table 3.1. Molecular masses of proteins identified from MALDI mass spectra and their identities based on molecular mass matching with *E. coli* proteome databases.

Mass from CZE-MS	Mass from LC-CZE-MS	Mass of Possible Match	Name and Description
	2494.2	2496	NUSA protein (fragment)
2578.9	2580.0	2579	Replication initiator protein (fragment)
2621.8		2621.78	Entericidin B
	2851.8	2852.13	Lysis protein
	2918.1	2918	Hypothetical 2.9 kDa protein (fragment)
	3143.9	3145.77	LEU operon leader peptide
	4138.3	4136.76	Heat-stable enterotoxin 1
	5707.7	5707	Z3951 protein
	5938.2	5936.84	GCV operon transcriptional regulator
	6029.8	6026.89	Hypothetical 6.0 kDa protein in AEFA-PRIC intergenic region
	7158.8	7157.74	50S ribosomal protein L35 (ribosomal protein A)
	7271.6	7271.17	Cold shock-like protein CSPC (CSP-C)
	7333.3	7333.54	Entry exclusion protein B (TRBK protein)
	7725.6	7726.97	YAIA protein
	7739.0	7739.39	ORF70
	7741.8	7741.01	Lysis protein S homolog from lambdoid prophage QIN
7746.3		7745.13	Heme exporter protein D (cytochrome C-type biogenesis protein CCMD)
	7923.2	7923.10	S protein
8224.9		8225	BFPA (fragment)
	8357.0	8359	Hypothetical 8.4 kDa protein
	8450.6	8449.89	Hypothetical 8.5 kDa protein L0053 (ORF2)
	9093.7	9094.54	Hypothetical 9.1 kDa protein in FTSY-NIKA intergenic region
	9113.2	9114.11	YHEA protein
9119.9		9119.37	Phosphocarrier protein HPR (histidine-containing protein)
	9754.1	9753.24	SLT-I B subunit
	9758.1	9755.8	Hypothetical 9.8 kDa protein in DING-GLNQ intergenic region
9767.4		9767.45	SSB24=(GAL4(1-147))-SSB24 transcriptional repressor component
	10342.5	10340.59/ 10344.46	Negative regulator of flagellin synthesis (anti-SIGMA-28 factor)/ hypothetical lipoprotein YBFN
	10528.0	10528.21	Hypothetical 10.5 kDa protein
	10530.3	10529.14	PERC
10534.8		10534.31	Hypothetical protein
	10752.4	10750.25/ 10753.68	Probable SIGMA(54) modulation protein (ORF3) (ORF95)/ hypothetical 10 8 kDa protein
	11828.4	11825	Hypothetical 11.8 kDa protein in IBPA-DGOT intergenic region (precursor)
	11976.5	11976.13	Protein YGIW
	12055.7	12055	Putative transcriptional regulator
15917.8	15917.4	15917.27	F200

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The experimental procedure for the LC pre-separation is described in Section 3.3.5. The entire process of elution and the collection of 19 fractions were completed in less than 5 minutes. MALDI-MS measurements were performed on these LC fractions to determine the presence of proteins. The proteins of interest, with molecular weights over 4000 Da, were detected in the thirteenth to the nineteenth fractions. The MALDI mass spectra of these seven fractions are shown in Figures 3.3A-G respectively. Figure 3.3O presents the mass spectrum of the raw extract prior to separation. The small differences in the spectra of Figures 3.2O and 3.3O are due to minor variations associated with the cell extraction and MALDI sample preparation – the relative peak intensities in MALDI spectra are very sensitive to the experimental conditions used.

A number of peaks are observed in Figures 3.3A-G. New proteins are also detected, such as the 10528 Da protein in fractions 13 and 14, which are not observed in Figure 3.3O. Detection of new proteins indicates the reduction of ion suppression in MALDI analysis. Some proteins are detected in only one fraction (e.g., 10753 Da in fraction E), and others appear in more than one fraction. Nevertheless, differences in the relative peak intensity are observed for the recurring proteins (e.g., between 10530 Da and 15917 Da in fractions A and B, and between 8450 Da and 10752 Da in fractions D and E). Hence, a crude separation by preparative LC is evident. The LC pre-separated fractions were subsequently injected for CZE separation.

3.4.3. CZE after LC Pre-separation

Prior to CZE injection, the seven LC fractions (A to G) were evaporated from 500 μL to *ca.* 5 μL to remove acetonitrile and concentrate the proteins. Injection times from 1.0 to 10.0 seconds were used for the various fractions depending on the amount of protein present. The electrophorograms from the seven LC fractions are shown in the insets of Figures 3.4-3.10.

All electrophorograms begin with the fronted TFA peak at *ca.* 2 minutes. For long injection times, the TFA peak broadens to a trapezoid-shaped signal as a result of serious injection band broadening. Following the TFA signal, protein peaks are detected in each sample up to 12 minutes. The protein peaks observed are still broad and not fully resolved. Although the sample was pre-separated by LC prior to CZE, presumably a similar degree of electrodispersion and injection band broadening remained. Hence significant improvement in peak efficiency is not observed. MALDI-MS analysis was then used to identify the CZE separated components and evaluate the performance of the CZE separation.

3.4.4. CZE Fraction Collection and MALDI-MS

Electrokinetic fraction collection was performed on each CZE separation. The number of fractions collected ranged from one up to eight depending on the signals observed on the electrophorogram. A MALDI mass spectrum was recorded for each of the CZE fractions

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collected, and these are shown in Figures 3.4-3.10. The time and duration of CZE fractionation are illustrated graphically on the inset electrophorograms.

In general, differences are observed in the MALDI spectra of different CZE fractions. In some cases, different proteins are identified from different fractions. When proteins recur in adjacent fractions, a shift in the relative intensity is always observed. LC pre-separation prior to CZE allows more proteins to be detected by MALDI. This is best illustrated by a comparison of the results obtained in Figure 3.9 to those in Figure 3.3F. The MALDI spectrum of LC fraction F shows a few protein peaks (Figure 3.3F), but many more are detected after CZE fractionation (Figure 3.9). Overall, separation by LC-CZE results in the detection of more proteins, including some that are not detected in the original mixture. The proteins identified from the mass spectra of LC-CZE fractions are listed in the second column of Table 3.1. The number of proteins identified after LC-CZE separation is more than three times that detected with CZE separation alone (column 1 of Table 3.1). This indicates that the LC-CZE separation reduces analyte interference in MALDI-MS, allowing detection of previously suppressed signals.

A final remark is necessary regarding the intensity of the mass spectra of CZE fractions. Lower signal intensities are observed from the spectra of CZE fractions (panels a, b, c, ... in Figures 3.4-3.10) compared to those of the LC fractions prior to CZE (Figures 3.3A-G). The MALDI spectra of the LC fractions in Figure 3.3 are obtained without any concentration or dilution. For the MALDI-MS of the CZE fractions, the LC fractions are concentrated 100-fold by evaporation prior to CZE injection. A hydrodynamic injection at 5 kPa is performed for 1.0 to 10.0 seconds, delivering from 3 to 30 nL of sample. After CZE, the separated components are collected into 4 μL of HCl solution. This results in significant dilution, for which even the initial sample concentration could not fully compensate. Hence weaker MS signals are expected from the CZE fractions. This becomes a problem for low concentration samples, such as fraction 19 (Figure 3.10). Many signals detected prior to CZE (Figure 3.3G) are lost after CZE and fractionation (Figure 3.10), even when an extended injection time (10.0 seconds) and reduced volume of HCl solution (2 μL) are used.

In addition to dilution, another source of protein loss is the adsorption of proteins onto surfaces. We found that the MALDI-MS measurement of fractions was best performed immediately after collection. Protein adsorption takes place on surfaces such as the vial, electrode and capillary, presumably via both electrostatic and hydrophobic interactions. Because the volume and concentration of protein in our samples are very low, the effect of protein loss is magnified. However, the adsorption problem associated with protein analysis is not specific to capillary electrophoresis or fraction collection. The strong tendency of hydrophobic proteins to adsorb onto surfaces applies to other techniques, such as HPLC and gel electrophoresis. Improved techniques in protein recovery after a separation will be investigated in the future.

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2.5. Conclusions

Separation of a complex protein mixture by CZE has been demonstrated. Partial separation is observed in a 10-minute run. LC is employed as a pre-separation to CZE to improve the resolution. LC-CZE provides good separation of proteins from *E. coli* cell extracts. It reduces analyte interference in MALDI-MS measurement, and increases the number of proteins identified by more than a factor of three. This work has also identified some important issues related to the development of LC-CE MALDI-MS as a practical tool for proteome analysis. Future work will focus on improving separation efficiencies in both LC and CE for complex protein mixture analysis, developing hardware for better LC to CE to MALDI-MS interfaces, and implementing enzymatic digestion of final fractions for protein identification based on peptide mass mapping and fragment ion spectra of peptides.

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2.7. Figures

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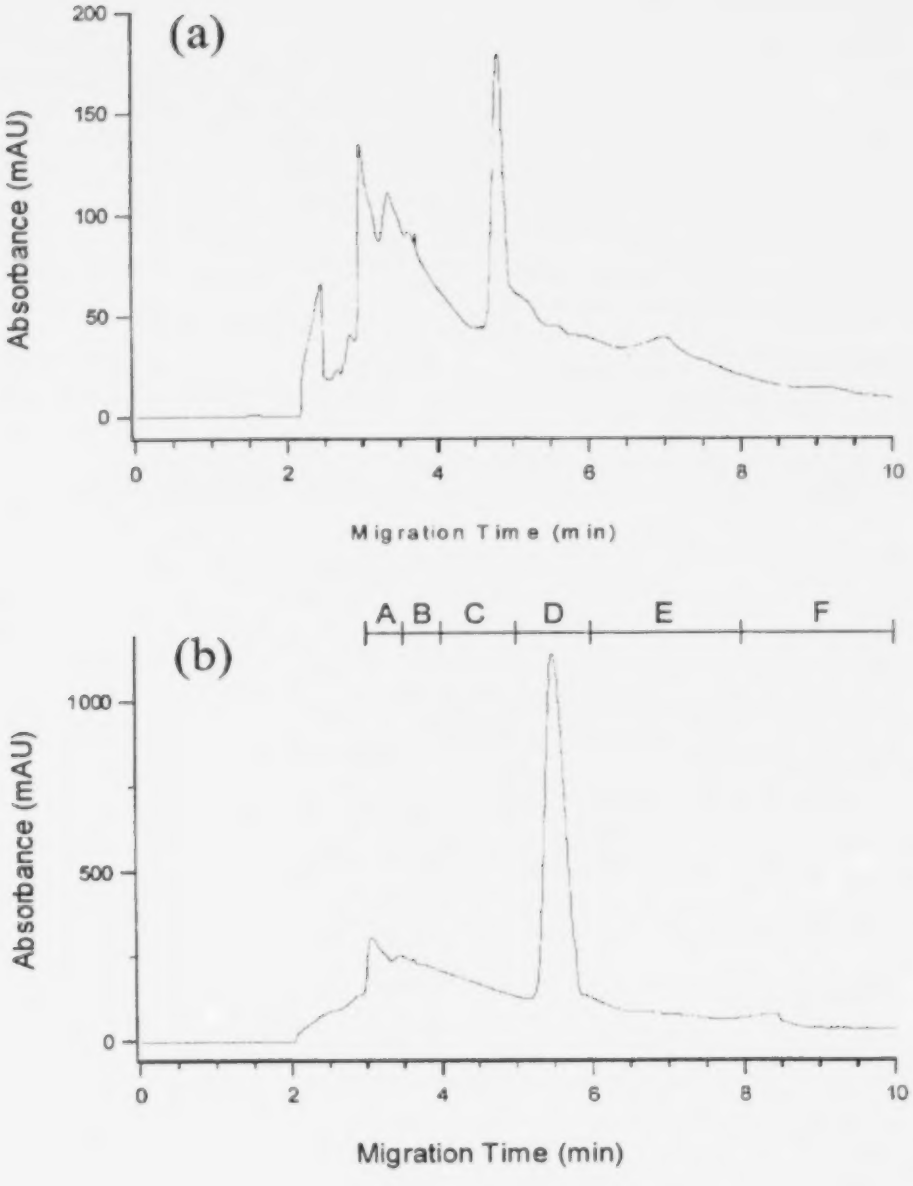

Figure 3.1. CZE separation of proteins from *E. coli* extracts. Experimental conditions: capillary length, 30.5 cm (22.3 cm to detector); separation voltage, -6 kV; injection, 1.0 second (A) and 5.0 seconds (B).

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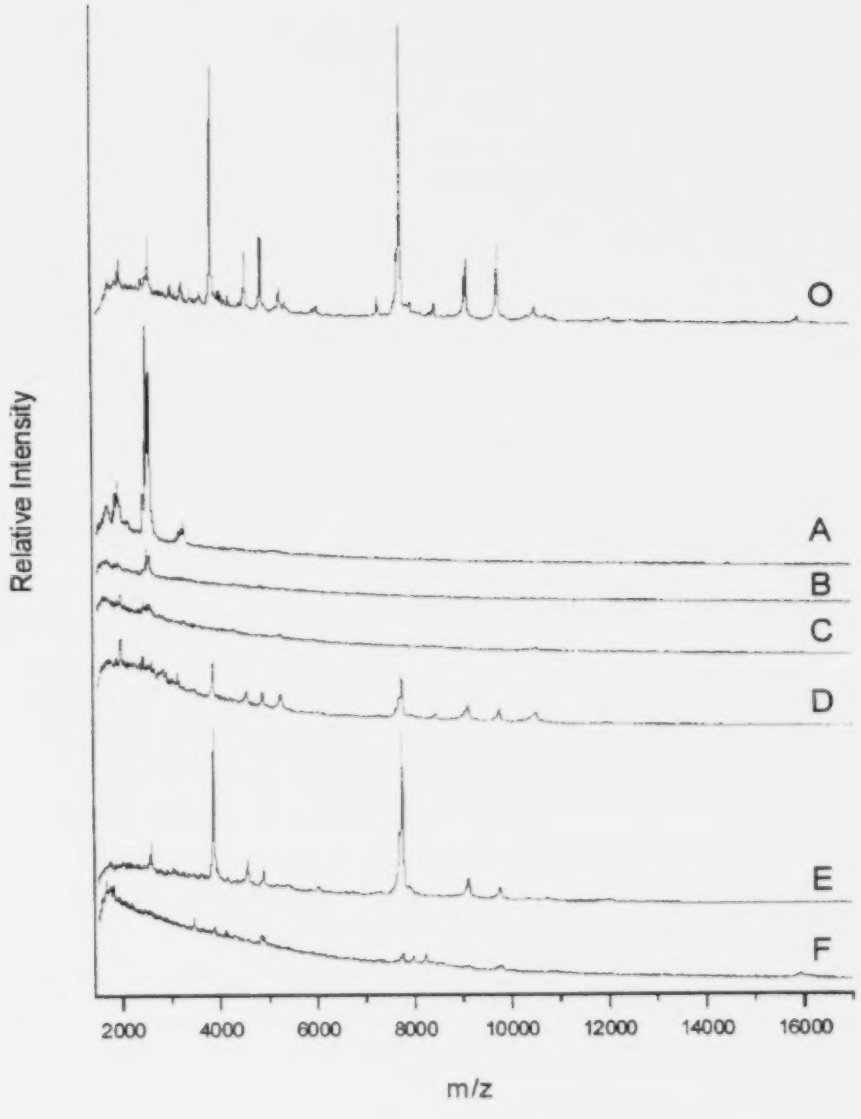

Figure 3.2. MALDI mass spectra of CZE fractions collected from Figure 3.1B (A-F) and from the original *E. coli* sample (O).

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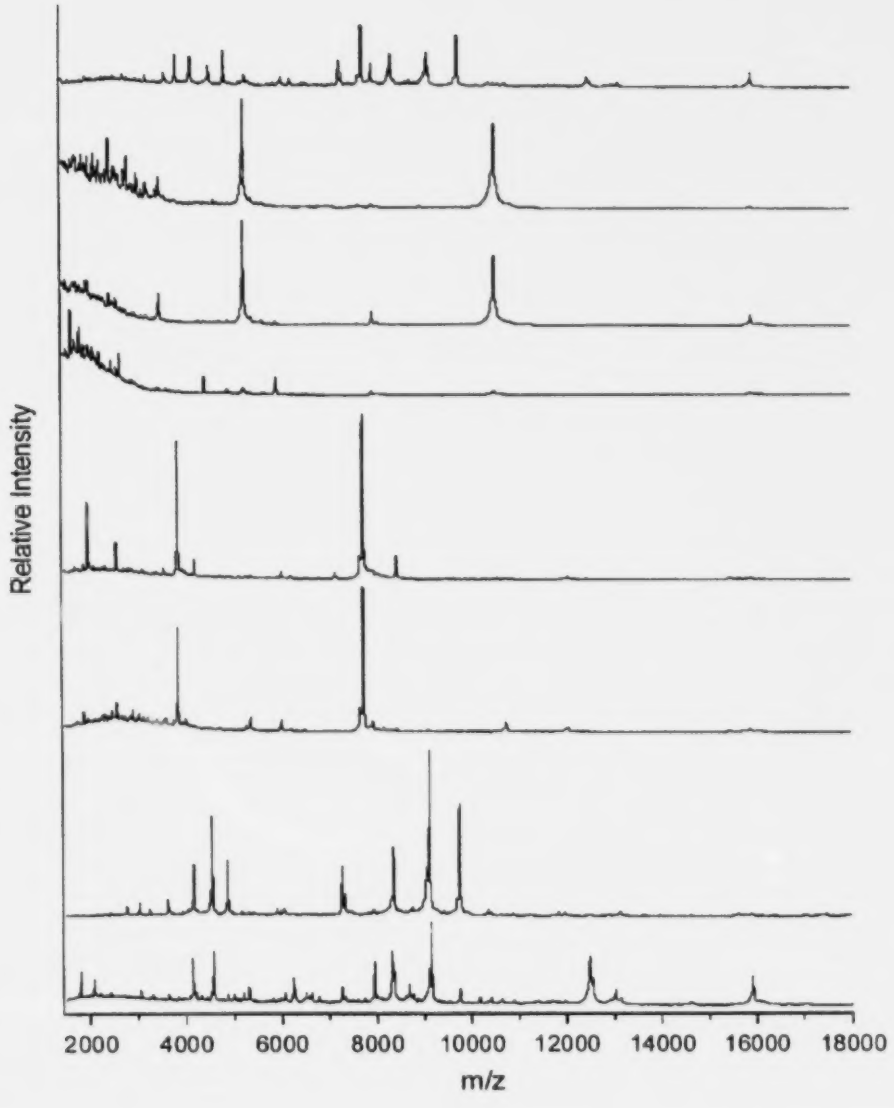

Figure 3.3. MALDI mass spectra of LC fractions (A-G) and of the original *E. coli* sample (O).

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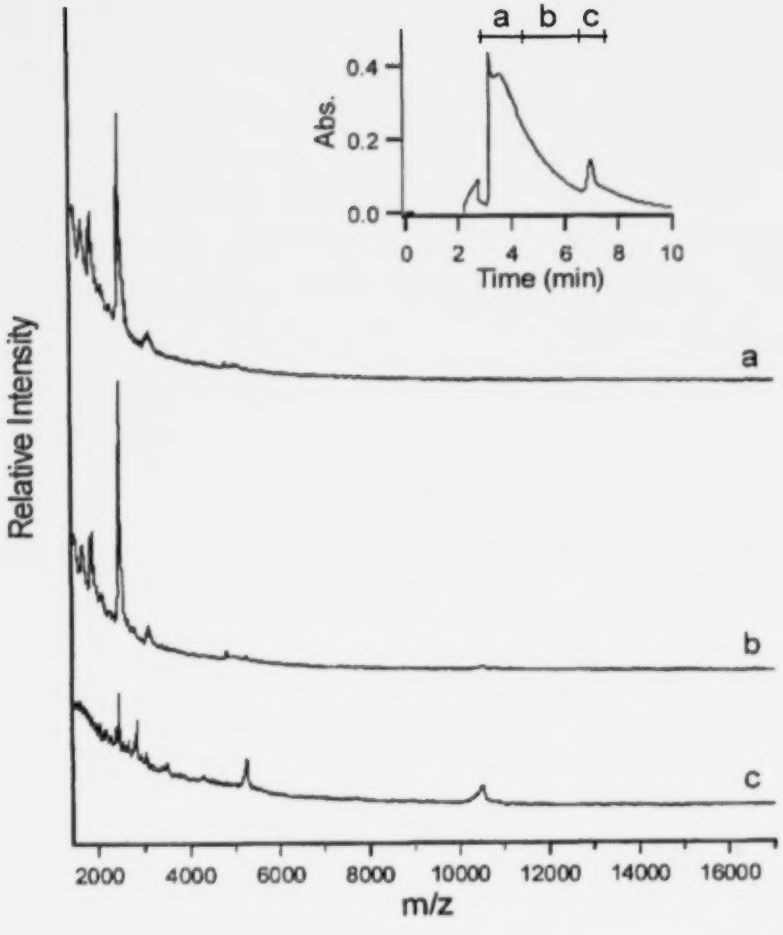

Figure 3.4. Electropherogram (inset) of LC fraction A and MALDI mass spectra of the corresponding CZE fractions.

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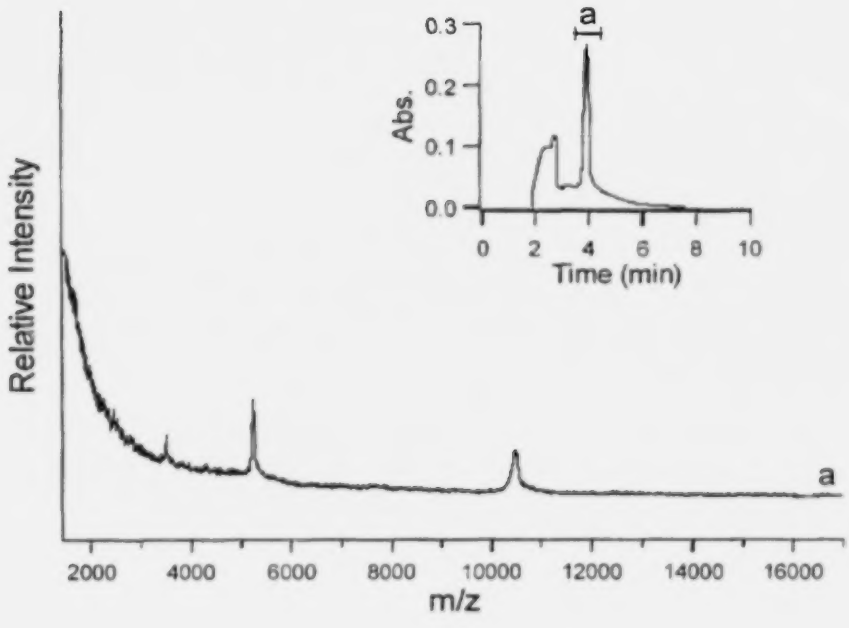

Figure 3.5. Electropherogram (inset) of LC fraction B and MALDI mass spectra of the corresponding CZE fractions.

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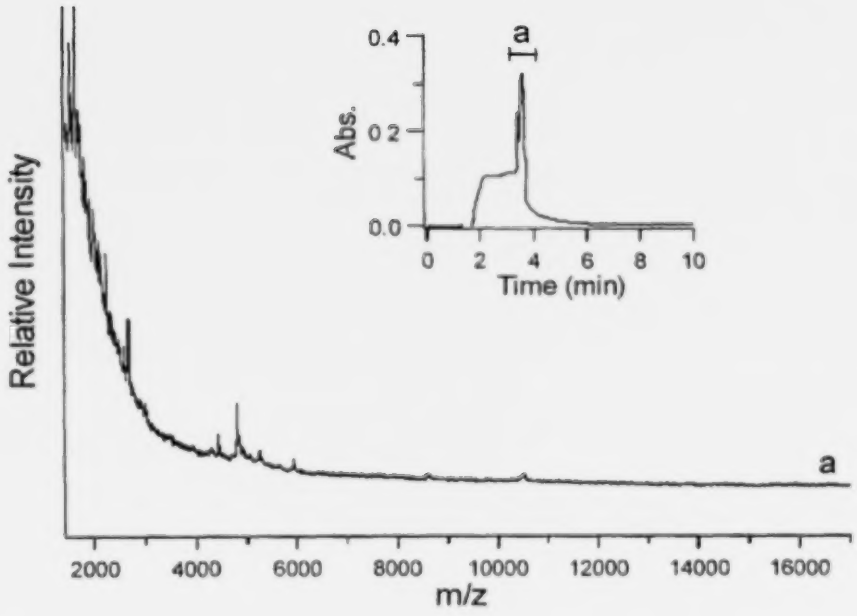

Figure 3.6. Electropherogram (inset) of LC fraction C and MALDI mass spectra of the corresponding CZE fractions.

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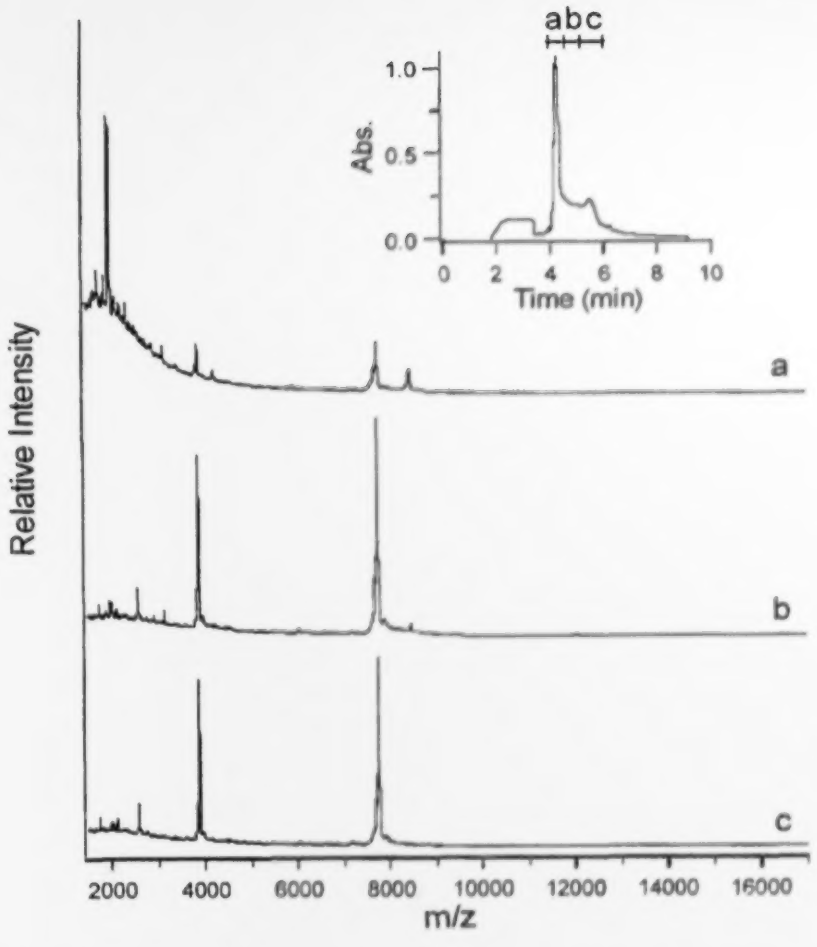

Figure 3.7. Electropherogram (inset) of LC fraction D and MALDI mass spectra of the corresponding CZE fractions.

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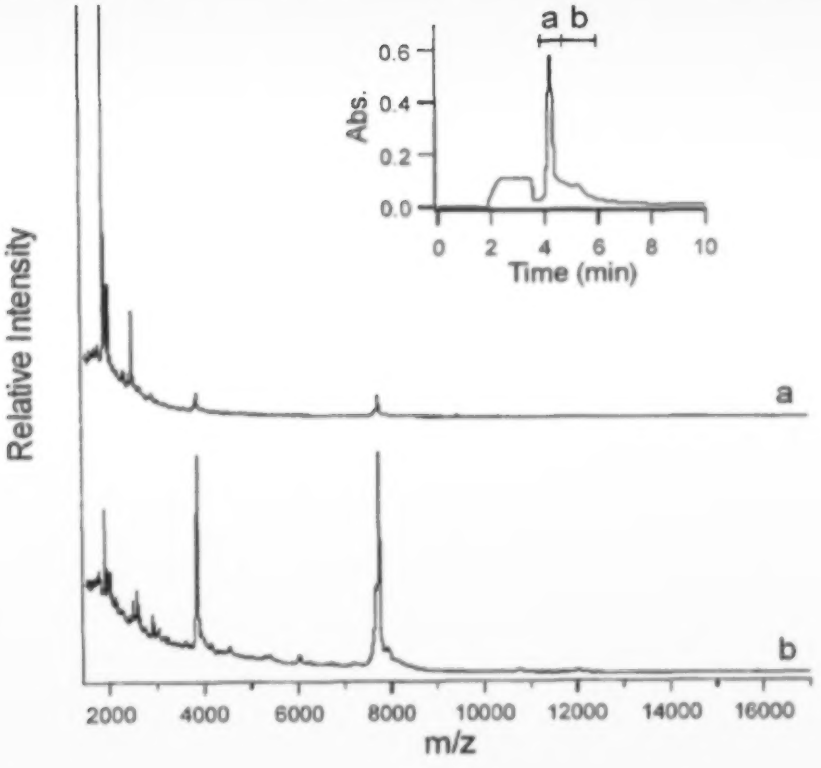

Figure 3.8. Electropherogram (inset) of LC fraction E and MALDI mass spectra of the corresponding CZE fractions.

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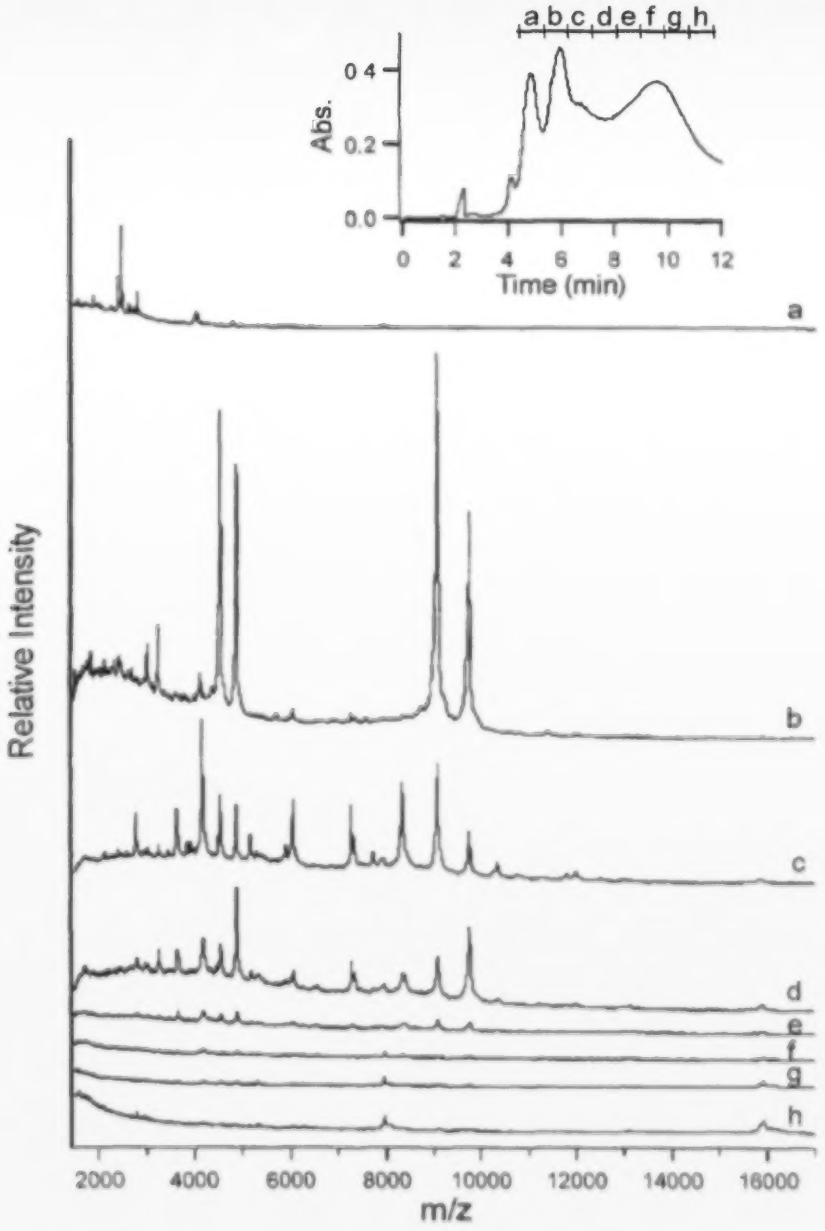

Figure 3.9. Electropherogram (inset) of LC fraction F and MALDI mass spectra of the corresponding CZE fractions.

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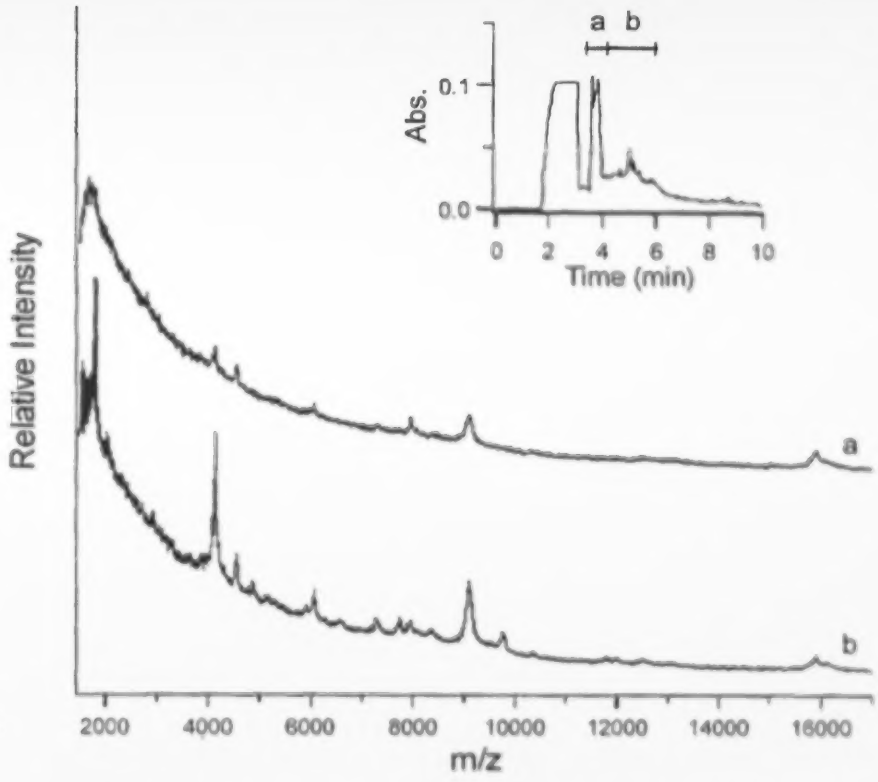

Figure 3.10. Electropherogram (inset) of LC fraction G and MALDI mass spectra of the corresponding CZE fractions.